Characterisation of methionine metabolism of *Pseudomonas aeruginosa* under conditions resembling a chronic cystic fibrosis lung infection

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“Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning.”

Albert Einstein

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Abbreviations

AAM	Amino acid medium
AAMM	Amino acid medium modified
ACh	ASM-Chelex
ACP	Acyl carrier protein
Ado	Adenosine
AHL	N-acylhomoserine lactone
AMC	Activated methyl circle
Anr	Anaerobic regulation of arginine deiminase and nitrate reduction
Ap^r	Ampicillin resistance
ASL	Airway surface liquid
ASM	Artificial sputum medium
ATP	Adenosine triphosphate
BHL	N-butyryl homoserine lactone
BICP	5-Brom-4-chlor-3-indoxyl phosphate
bp	Base pair(s)
BSA	Bovine serum albumin
°C	Degree Celsius
^{13}C	Carbon-13, stable isotope of carbon
CAMP	Cationic antimicrobial peptide
c-di-GMP	Cyclic di-guanylate
cDNA	Copy DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CM	Cytoplasmic membrane
Da	Dalton
DNA	Deoxyribonucleic acid
Dnr	Dissimilatory nitrate respiration regulator
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTPA	Diethylene triamine pentaacetic acid
E4P	Erytrose-4-phosphate
ECF sigma factor	Extracytoplasmatic function sigma factor
EV	*Ex vivo*
FRT	Flp recombinase target
Fur	Ferric uptake regulator
F_{xxx}	Relative Fluorescence at xxx nm
g	Centrifugation: earth gravity (x g); weight: gram (g)
Gm^r	Gentamycin resistance
GO	Guanine 8-oxo-2-deoxyguanosine

h	Hour(s)
Hcy	L-homocysteine
His-	Histidine tagged
IPTG	Isopropyl β-D-1-thiogalactopyranoside
l	Liter
LPS	Lipopolysaccharide
M	Molar [Mol /l], or mega in Mbp
m	Milli
MCC	Mucocilliary clearance
MCS	Multiple cloning site
MDR	Multi-drug resistant
Me-	Methyl-
Met	L-methionine
min	Minute
MMR	Methyl-directed DNA mismatch repair
n	Nano
NBT	Nitro blue tetrazolium
OM	Outer membrane
odDHL	N-3-oxododecanoyl homoserine lactone
OD_{xxx}	Optical density at xxx nm
Pa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction(s)
PMF	Proton motive force
p-value	Probability value
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAH	S-adenosyl-L-homocysteine
SahH	S-adenosylhomocysteine hydrolase
SAM	S-adenosyl-L-methionine
SNP	Single-nucleotide polymorphism(s)
T3SS	Type three secretion system
TCA	Tricarboxylic acid cycle
THF	Tetrahydrophosphate
THP	Tetrahydropteroyltriglutamate
TNB^{2-}	5-thio-2-nitrobenzoate
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet
vs.	Versus
wt	Wild type

Zusammenfassung

Im ersten Teil dieser Arbeit wurde der Einfluss der Wachstumsbedingungen in den zystische Fibrose (CF) Luftwegen auf die infizierenden *Pseudomonas aeruginosa* Stämme untersucht. Eine globale Transkriptionsanalyse der *P. aeruginosa* Genexpression wurde sowohl unter simulierten Respirationstraktbedingungen in artifiziellem Sputum-Medium (ASM) als auch unter *ex vivo* CF Lungeninfektionsbedingungen durchgeführt. Dabei wurde Eisenmangel als der entscheidende Faktor in den CF Luftwegen *in vivo* identifiziert. Daraufhin wurde ASM-Chelex mit erhöhter Eisenlimitierung entwickelt. Interessanterweise scheint die transkriptionelle Antwort auf Eisenlimitierung eine generelle Antwort von *P. aeruginosa* auf Infektionsbedingungen zu sein. Unter *ex vivo* Bedingungen waren die *P. aeruginosa* Stämme metabolisch sehr inaktiv. Die Geneexpression deutet auf die Nutzung der Denitrifikation zur Engergieerzeugung während mikroaerober bis anaerober Wachstumsbedingungen *ex vivo* hin. Cholin-, Methionin- und SAM-Stoffwechsel wurden als wichtige Faktoren unter *ex vivo* Bedingungen ermittelt. Unerwarteterweise wurden Flagellen und Typ-III-Sekretionssystem (T3SS) Gene unter *ex vivo* Bedingungen exprimiert gefunden. Zusammenfassend imitiert ASM-Chelex die *in vivo* Lungeninfektionsbedingungen präziser.

Im zweiten Teil wurde die Methioninauxotrophie als Anpassungsstrategie von *P. aeruginosa* an die chronische CF Lungeninfektion untersucht. Insgesamt wurden 30 *P. aeruginosa* Stämme von 15 verschiedenen CF Patienten isoliert und durch Einzelnukleotid-Polymorphismen (SNP) klassifiziert. Als genetische Ursache für die Methioninauxotrophie wurde das Gen *metF* der Kofaktorbiosynthese für die Mehrheit der untersuchten klinischen Isolate identifiziert. Das Gen *metF* kodiert für eine Methylentetrahydrofolat-Reduktase. Es wurde bewiesen, dass die Methioninauxotrophie unabhängig vom Mutatorphänotyp selektioniert wird. Die Produktion des bedeutenden Virulenzfaktors Pyocyanin war in allen methioninauxotrophen klinischen Isolaten, in PAO1 Δ*metF* und in verschiedenen Methioninstoffwechseldeletionsmutaten reduziert. Als Grund wurde der mögliche verringerte SAM-Spiegel diskutiert. Die Transkriptomanalyse von PAO1 Δ*metF* zeigte, dass eisenregulierte Gene stärker exprimiert wurden. Der Einfluss der Methioninauxotrophie auf die Eisenhomöostase und mögliche Eisenbeschaffungsvorteile für PAO1 Δ*metF* wurde diskutiert.

Im dritten Teil wurde die antimikrobielle Aktivität von Ribavirin gegen *P. aeruginosa* PAO1 nachgewiesen. Die S-Adenosylhomocystein-Hydrolase SahH scheint nicht der Hauptangriffspunkt von Ribavirin in *P. aeruginosa* zu sein. Die Inhibition des Enzyms SahH durch Ribavirin war sehr gering. Es wurde jedoch gezeigt, dass das Enzym SahH ein essentieller Bestandteils des bakteriellen Stoffwechsels ist und sich daher besonders gut als Ziel für Antibiotika eignet. Eine Deletion des *sahH* Genes hat drastischen Auswirkungen auf das bakterielle Wachstum. Der unausgeglichene intrazelluläre SAH-/SAM-Spiegel wurde als möglicher Grund diskutiert.

Diese Arbeit trägt zum Verständnis der zugrunde liegenden Anpassungsstrategien von *P. aeruginosa* während der CF Infektion bei und zeigt mögliche therapeutische Strategien auf.

Summary

The first part of this study investigated the influence of the growth conditions in the cystic fibrosis (CF) airways on the infecting *Pseudomonas aeruginosa* strains. A global transcriptional analysis of *P. aeruginosa* gene expression under simulated respiratory tract conditions *in vitro* in artificial sputum medium (ASM) and under *ex vivo* CF lung infection conditions was performed. Iron limitation was identified as the crucial factor in the CF airways *in vivo.* Therefore, ASM-Chelex with increased iron limitation was developed. Interestingly, the transcriptional answer to iron limitation seems to be a general answer of *P. aeruginosa* towards infection conditions.
Under *ex vivo* conditions *P. aeruginosa* strains were metabolically very inactive. The gene expression indicates the use of denitrification for energy generation during microaerobic to anaerobic growth conditions *ex vivo*. Choline, methionine and SAM metabolism were determined as important factors during *ex vivo* conditions. Unexpectedly, flagella and Type three secretion system (T3SS) genes were detected upregulated under *ex vivo* conditions. In conclusion ASM-Chelex mimics *in vivo* lung infection conditions more precisely.

In the second part methionine auxotrophy as adaptation strategy of *P. aeruginosa* towards the chronic CF lung infection was investigated. In total 30 *P. aeruginosa* strains isolated from 15 different CF patients were classified by single-nucleotide polymorphisms analysis (SNP). As genetic cause of methionine auxotrophy the gene *metF* of the cofactor biosynthesis was identified in the majority of investigated clinical isolates. The gene *metF* encodes for a methylenetetrahydrofolate reductase. It was verified that the methionine auxotrophy is selected independently of the mutator phenotype. Notably, the production of the major virulence factor pyocyanin was reduced in all methionine auxotrophic clinical isolates, in PAO1 Δ*metF* and several methionine metabolism deletion strains. The possible reduced intracellular SAM level was discussed as cause. A transcriptome analysis of PAO1 Δ*metF* showed upregulation of iron regulated genes. The influence of methionine auxotrophy on iron homeostasis and the possible advantage in iron acquisition of PAO1 Δ*metF* was discussed.

In the third part the antimicrobial activity of ribavirin against *P. aeruginosa* PAO1 was verified. S-Adenosylhomocysteine hydrolase SahH seems not to be the main target of ribavirin in *P. aeruginosa*. The inhibition of the enzyme SahH by ribavirin was very low. However, it was shown that the enzyme SahH is an essential part of bacterial metabolism and therefore a powerful drug target candidate. The deletion of the *sahH* gene has drastic effects on bacterial growth. The unbalanced intracellular SAH- / SAM-level was discussed as possible reason.

In summary this thesis contributes to the understanding of the underlying adaptation strategies of *P. aeruginosa* during the CF infection and indicates possible therapeutic strategies.

1 Introduction

The need for new antibiotic substances and new antibiotic targets is undeniable especially in the times of multi-drug resistant (MDR) bacteria. Antibiotics target essential life processes such as cell-wall biosynthesis and the cell envelope, protein biosynthesis, RNA and DNA replication and the folate metabolism (Walsh, 2003).

Treatment of MDR bacteria is particularly difficult during the reoccurring bacterial lung infections of cystic fibrosis (CF) patients. CF is the most common lethal genetic disease in the white population (O'Sullivan and Freedman, 2009).

Resistance against commonly used CF antibiotics such as tobramycin and ciprofloxacin is increasing in *Pseudomonas aeruginosa*, therefore new therapeutical approaches, new effective substances and new targets are necessary (Amini *et al.*, 2011; Brazas *et al.*, 2007; Wu *et al.*, 1999). In this context it is essential to understand the underlying conditions of the chronic *P. aeruginosa* CF lung infection.

1.1 Cystic fibrosis and *Pseudomonas aeruginosa* infection

CF is a recessive autosomal hereditary disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene with a prevalence of 1 in 2500 live births in populations of European ancestry (Kerem *et al.*, 1989; O'Sullivan and Freedman, 2009; Riordan *et al.*, 1989; Rommens *et al.*, 1989). Approximately 70 000 children and young adults are affected worldwide with the majority of cases in the European Union and in the United States (Becker *et al.*, 2010). The prevalence in Africa and Asia is much less with 1 in 15 000 to 20 000 and 1 in 350 000 in Japan, respectively (O'Sullivan and Freedman, 2009). The median life expectancy in 1969 was 8 years. Due to constant antibiotic treatment and improvements in medical therapy the median life expectancy for patients born in the 2000s will likely be 40 to 50 years (Folkesson *et al.*, 2012; Foundation, 2013; O'Sullivan and Freedman, 2009).

Currently, 1500 possible mutations in the *CFTR* gene are described (O'Sullivan and Freedman, 2009). The most common mutation is ΔF508 a deletion of phenylalanine 508 (Jaffe and Bush, 2001). The different mutations result in a loss-of-function or malfunction of the CFTR protein, which mainly operates as chloride ion channel besides other possible functions (Darling *et al.*, 2004; Roomans, 2003). CFTR is described to have regulatory functions in ATP and chloride ion channels, in inhibition of sodium transport through the epithelial sodium channels and in inhibition of calcium-dependent chloride ion channels (O'Sullivan and Freedman, 2009). Moreover, CFTR is important for regulation of intracellular vesicle transport and acidification of intracellular organelles (O'Sullivan and Freedman, 2009). CFTR is present on most epithelial surfaces and at the surface of blood cells (O'Sullivan and Freedman, 2009).

The impaired CFTR function results in thick dehydrated mucus in all exocrine glands particularly of the respiratory, the gastrointestinal and the reproductive tract (Hogardt and Heesemann, 2013; Riordan *et al.*, 1989). Depending on the type of mutation and the severity of the condition the clinical symptoms vary among patients (Hauser *et al.*, 2011; Lyczak *et al.*, 2002). Different hypotheses exist trying to explain the underlying reasons for the high viscosity of mucus in the CF airways (O'Sullivan and Freedman, 2009; Verkman *et al.*, 2003). Besides, the mutation in the *CFTR* gene, a variety of innate immune system dysfunctions have been reported, which all

together lead to the chronic bacterial infection of the lung and excessive pulmonary inflammation (Doring and Gulbins, 2009; Hogardt and Heesemann, 2013).

The mucocilliary clearance (MCC), the cationic antimicrobial peptides (CAMPs), the CFTR, and moreover, neutrophils and macrophages are impaired (Doring and Gulbins, 2009) (Fig. 1 A&B). Recently, ceramide accumulation as effect of CFTR mutation was discovered (Becker *et al.*, 2010; Teichgräber *et al.*, 2008).

The MCC is impaired due to the high viscosity of the mucus compared to the thin airway surface liquid (ASL) of the healthy lung (Fig. 1 A&B) (Boucher, 2007). This condition allows bacterial pathogens to colonise the nutrient rich mucus. The mucus is rich in amino acids of degraded proteins, glycoproteins (e.g. mucins), DNA, phospholipids, electrolytes, cellular debris from neutrophils and bacteria (Barth and Pitt, 1996; Palmer *et al.*, 2005; Whitchurch *et al.*, 2002). Nitrate concentrations up to 700 µM were detected in the CF sputum (Grasemann *et al.*, 1998).

The release of bacteriolytic enzymes and CAMPs such as β-defensins and cathelicidins is probably reduced due to the high viscosity of mucus (Verkman *et al.*, 2003). Additionally, the probably hypertonic salt concentrations in the mucus might inactivate the salt-sensitive CAMPs (Boucher, 2007).

Furthermore, CFTR was shown to be responsible for the internalization of *P. aeruginosa* and is therefore essential for clearance of *P. aeruginosa* from the epithelial surface (Pier, 2000). The high viscosity of mucus also influences migration of neutrophils (Matsui *et al.*, 2005), which results in delayed eradication of pathogens and therefore in higher bacterial cell numbers (Fig. 1B). Besides bacteria have more time to adapt to the environment.

In contrast the neutrophil levels in the CF lung are 380-fold higher than in the lungs of the uninfected controls (Konstan *et al.*, 1994). In addition, the neutrophil activation results in the release of granule stored enzymes such as neutrophil elastase, myeloperoxidase and in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Hogardt and Heesemann, 2013). The imbalance between protease / antiprotease and oxidants / antioxidants activity results in further tissue damage (Doring, 1999; Ratjen *et al.*, 2002). Additionally, neutrophil necrosis enhances extracellular DNA concentrations.

Recently, it was demonstrated that CFTR in the lysosome participates in pH control. Since the chloride ion transport by CFTR positively stimulates the hydrogen ion transport into the lysosomal lumen by the V-type H^+-ATPase (Di *et al.*, 2006). This results in a pH of < 5 which is necessary for optimal bactericidal activity of degenerative enzymes during fusion of the lysosome with the phagosome.

A defective CFTR results in increased pH. The elevated pH causes an imbalance of acid sphingomyelinase and acid ceramidase. Ceramide accumulation is the consequence. The cell death rates are increased resulting in extracellular DNA deposition (Teichgräber *et al.*, 2008).

An increase in proinflammatory molecules such as interleukin 8, interleukin 6 and tumor necrosis factor α has been detected in CF patients (Colombo *et al.*, 2005). Nuclear factor-κB pathway, platelet hyper-reactivity, and abnormalities in neutrophil apoptosis have also been reported (O'Sullivan and Michelson, 2006; Rottner *et al.*, 2007). Native anti-inflammatory substances such as interleukin 10, lipoxin, and docosahexaenoic acid are reduced, leading to

an imbalance between proinflammatory and anti-inflammatory response. This imbalance leads to undiminished inflammation (Bonfield *et al.*, 1999).

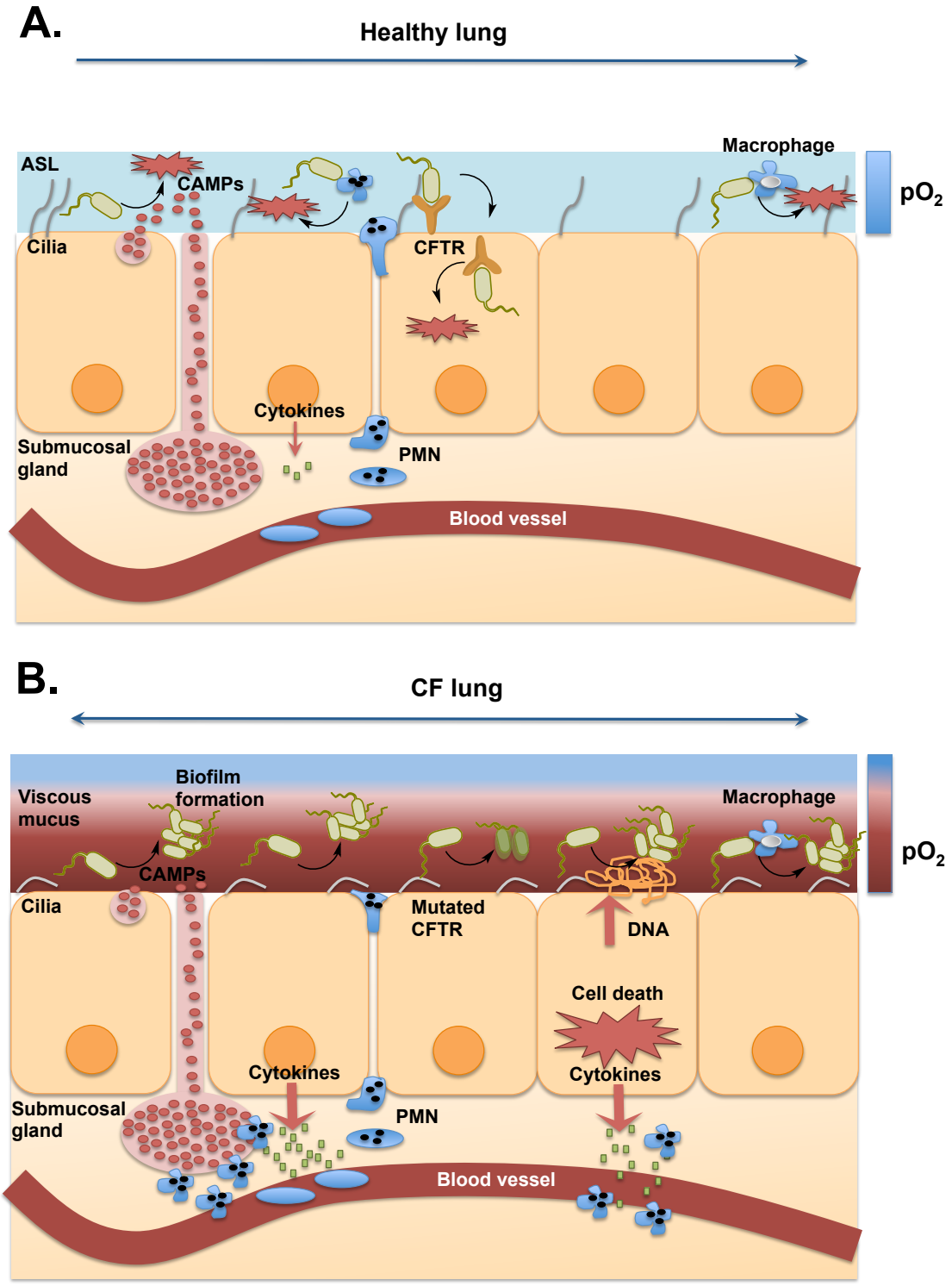


Fig. 1: Clearance of bacteria from the airways of healthy individuals and cystic fibrosis patients (adapted from Doring and Gulbins, 2009 and Worlitzsch *et al.*, 2002)

The clearance of bacterial airway infections is shown for healthy individuals (A) and cystic fibrosis (CF) patients (B).

(A) Bacteria reaching the respiratory tract of healthy individuals are efficiently trapped by the thin airway surface liquid (ASL) layer, which is constantly removed by the mucociliary clearance (MCC). Bacteria are killed by the cationic antimicrobial peptides (CAMPs) released from the submucosal glands or from epithelial cells. Eradication of bacteria can

take place via internalization by a functional CFTR or via uptake by neutrophils or macrophages. Neutrophils can eliminate bacteria by the formation of reactive oxygen or nitrogen species (ROS; NOS) or non-oxidative by CAMPs. The conditions in the ASL are aerobic.

(B) In the respiratory tract of CF patients bacteria can survive in the highly viscous nutrient rich mucus, due to impaired MCC and impaired CAMPs release. The migration of neutrophils and macrophages is decreased. Depending on the type of *CFTR* gene mutation CFTR is not functional or not present on the epithelial surface at all. Therefore, no uptake of bacteria takes place. Due to the CTFR induced pH shift in the lysosomes of epithelial cells, neutrophils and macrophages bacterial pathogens are not killed in the phagolysosomes. Instead the pH shift and CFTR lead to ceramide accumulation, which results in cell death and DNA deposition. In addition, cytokine release leads to further neutrophil recruitment. The mucus is microaerobic to anaerobic, which induces phenotypic changes in the colonizing pathogens e.g. microcolony and biofilm formation by *P. aeruginosa* and the conversion to the mucoid phenotype. The oxygen gradient (pO_2) in the ASL and in the mucus is indicated with blue for oxygen saturation and red for oxygen depletion.

The microaerobic to anaerobic conditions in the CF airways (Worlitzsch *et al.*, 2002) cause the abolishment of the generation of reactive oxygen species (ROS) by the neutrophils and macrophages. Oxygen depletion is probably due to the high viscosity of the mucus, rapid bacterial oxygen consumption and probably also due to increased oxygen consumption by the epithelial cells (Stutts *et al.*, 1986; Worlitzsch *et al.*, 2002). Phagocytosis of *P. aeruginosa* is prevented by the mucoid phenotype and biofilm formation (Worlitzsch *et al.*, 2002). Moreover, alginate protects bacteria from and the effects of non-oxidative killing by antimicrobial peptides and other granule-stored enzymes (Hampton *et al.*, 1998; Matsui *et al.*, 2006; Worlitzsch *et al.*, 2002). Similarly, *Staphylococcus aureus* is producing a polysaccharide capsule, which protects from non-oxidative killing (McKenney *et al.*, 1999). *Burkholderia cepacia* complex is intrinsically resistant to non-oxidative killing (Sousa *et al.*, 2007).

Infants with CF are rapidly infected with *Haemophilus influenzae* or *S. aureus,* or both organisms. Approximately 80 % of CF patients are positive for *P. aeruginosa* in their late twenties (Hauser *et al.*, 2011). The airways of CF patients can be colonized by other pathogens besides *P. aeruginosa*, such as e.g. *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, methicillin-resistant *S. aureus* (MRSA), atypical mycobacteria and *Aspergillus fumigatus* (Hauser *et al.*, 2011; O'Sullivan and Freedman, 2009). Also anaerobic organisms were detected mostly belonging to the genera *Prevotella*, *Veillonella*, *Propionibacterium* and *Actinomyces* (Tunney *et al.*, 2008).

As described CF airways colonizing *P. aeruginosa* are confronted with a highly selective and heterogeneous environment, which is characterised by various stress conditions such as antibiotics, host immune response and oxidative and osmotic stress (Folkesson *et al.*, 2012).

Once a chronic *P. aeruginosa* airway infection is established, the infection is nearly impossible to cure and often associated with the decline of lung function and a reduced life expectancy (Doring and Gulbins, 2009; Hauser *et al.*, 2011).

1.1.1 Treatment of cystic fibrosis patients

At present the surveillance for *P. aeruginosa* is the most common strategy in combination with early eradication by inhaled antibiotics and with or without oral quinolone therapy (O'Sullivan and Freedman, 2009).

Currently, treatment with inhaled recombinant human deoxyribonuclease I and inhaled tobramycin is beneficial in many patients. The Cystic Fibrosis Foundation guidelines also support the use of inhaled hypertonic saline, the macrolide azithromycin and ibuprofen, in specific patient populations (Foundation, 2013; O'Sullivan and Freedman, 2009). Intravenous colistin has been found to be beneficial in the acute setting when combined with other antipseudomonal antibiotics (Foundation, 2013; O'Sullivan and Freedman, 2009). Most patients with exacerbations are most likely infected with *P. aeruginosa* and are therefore treated with a combination of β-lactams and aminoglycosides during in-hospital therapy.

1.2 Pseudomonas aeruginosa

1.2.1 The bacterium *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitously spread versatile Gram-negative bacterium, which is polar flagellated and belongs to the group of γ-proteobacteria (Stover *et al.*, 2000). Very characteristic for *P. aeruginosa* is the production of the blue pigment pyocyanin, which is known as major virulence factor in different infection models (Lau *et al.*, 2004), and the green fluorescent siderophore pyoverdin (Meyer, 2000; Meyer *et al.*, 1996; Schalk and Guillon, 2012). *P. aeruginosa* produces the volatile molecule 2-aminoacetophenone (Cox and Parker, 1979). This substance is responsible for the grape like sweet odour of *P. aeruginosa* cultures. Recently, 2-aminoacetophenone was described to have immuno modulatory effects, which influence the host signaling towards the establishment of a chronic infection in mice (Bandyopadhaya *et al.*, 2012).

Stover *et al.* (2000) determined the relatively large genome of *P. aeruginosa* PAO1 with a size of 6.3 Mbp and with currently 5671 predicted open reading frames (Winsor *et al.*, 2011). In addition Stover *et al.* (2000) observed that *P. aeruginosa* nearly possesses 300 cytoplasmic membrane transport systems. The majority of them seem to be involved in import of nutrients. This is in accordance with its enormous versatility in the environment.

Notably, 9,4 % of the open reading frames encode regulatory genes and two-component systems (Stover *et al.*, 2000). This number is much higher than in other bacterial species such as, *Escherichia coli* or *Bacillus subtilis* and enables *P. aeruginosa* to respond to a variety of changing environmental conditions (Stover *et al.*, 2000). The genome size of other so far sequenced *P. aeruginosa* strains ranges from 5 - 7 Mbp suggesting adaptations to different environmental niches (Schmidt *et al.*, 1996).

P. aeruginosa is capable to degrade various carbon sources, e.g. such as sugars, fatty acids, alcohols, glycols, aromatic compounds, amines and also amino acids (Palleroni, 1992; Palmer *et al.*, 2005; Son *et al.*, 2007). For the degradation of glucose *P. aeruginosa* uses the Entner-Doudoroff-pathway, which also favours the degradation of organic acids (Palleroni, 1992).

P. aeruginosa prefers as facultative anaerobe oxygen as terminal electron acceptor. Growth of *P. aeruginosa* under anaerobic conditions can be supported by either nitrate or nitrite respiration and by arginine fermentation (Hassett *et al.*, 2009; Vander Wauven *et al.*, 1984; Zumft, 1997). Anaerobic respiration consists of the sequential reduction of the alternative electron acceptor nitrate (NO_3) via nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O) to molecular nitrogen (N_2) (Zumft, 1997). Anaerobic survival without growth is possible via pyruvate fermentation (Eschbach *et al.*, 2004).

In addition, *P. aeruginosa* is able to form biofilms. Biofilms are structured communities of surface adherent bacteria covered in a polymeric matrix (Costerton *et al.*, 1999). Biofilm formation is important for the survival in various environments and during infection.

P. aeruginosa is known as opportunistic and nosocomial pathogen causing infections of the urinary tract, of burn wounds and particularly infections in immunocompromised patients, due to its intrinsic antibiotic resistances (Bielecki *et al.*, 2008; Chugani and Greenberg, 2007; Hancock,

1998). Furthermore, acute and persistent infections caused by *P. aeruginosa* are common in the lungs of CF patients (Hauser *et al.*, 2011; Lyczak *et al.*, 2002).

1.2.2 Quorum sensing and virulence in *Pseudomonas aeruginosa*

Quorum sensing regulates the expression of approximately 10 % of all genes in *P. aeruginosa* (Heurlier *et al.*, 2006). This includes genes encoding for functions such as virulence, motility, chemotaxis and metabolism. The quorum sensing network of *P. aeruginosa* is subdivided in the LasI / LasR and RhlI / RhlR system, which interact with the Pseudomonas quinolone signal (PQS) system, several global regulators, two-component systems and sigma factors (Fig. 2). The LasI / LasR system is regulating the RhlI / RhlR system and PQS production (Venturi, 2006).

The LasI / LasR and RhlI / RhlR system use the N-acyl-homoserine lactones (AHL) as signalling molecules. N-butyryl homoserine lactone (BHL) is synthesized by RhlI and N-3-oxododecanoyl homoserine lactone (odDHL) by LasI. Both AHLs synthases are constitutively expressed.

The transcriptional regulator LasR / odDHL positively regulates *lasI* gene and *rhlR* expression (Seed *et al.*, 1995). LasR forms multimers and binds in the presence of odDHL to las-boxes in the genome (Kiratisin *et al.*, 2002). RhlR dimerizes and binds DNA in the presence and absence of BHL (Medina *et al.*, 2003). Several global regulators have been identified to influence and regulate quorum sensing in *P. aeruginosa* (Venturi, 2006) (Fig. 2). This allows *P. aeruginosa* to regulate gene expression growth phase dependent, in regard to BHL and odDHL concentration and in accordance to the environmental conditions.

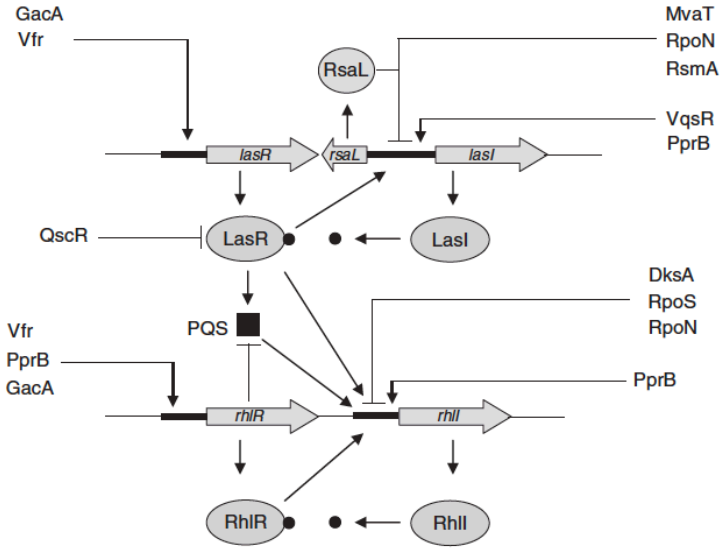


Fig. 2: Quorum sensing and regulatory network of *P. aeruginosa* (from Venturi, 2006)

Arrows indicate positive regulation. The short parallel lines show negative regulation.

Additionally, cyclic di-guanylate (c-di-GMP) seems to be important for swarming, twitching, surface attachment and biofilm formation. Accumulation of c-di-GMP promotes biofilm formation of *P. aeruginosa*, while its breakdown results in motility and detachment from biofilm towards planktonic lifestyle (Lory *et al.*, 2009).

The LasI / LasR quorum sensing system regulates the expression of the virulence factors such as elastase LasB, staphylolytic protease LasA, Exotoxin A and components of Xcp secretion machinery (Hentzer and Givskov, 2003; Juhas *et al.*, 2004; Schuster *et al.*, 2003; Wagner *et al.*, 2003). The RhlI / RhlR system regulates its own expression via RhlI. Moreover, the expression of sigma factor RpoS, rhamnolipid encoded by *rhlAB* operon, LasB, LasA, hydrogen cyanide encoded by the *hcnABC* operon, pyocyanin encoded by *phzABCDEFG* operon, lipase and alkaline protease AprA are regulated by the RhlI / RhlR system. PQS also regulates its own production by expression of the *pqsABCDE* operon and via PqsR (Dekimpe and Deziel, 2009). Moreover, PQS regulates the expression of elastase, rhamnolipid, PA-IL lectin LecA and pyocyanin (Diggle *et al.*, 2007b; Gallagher *et al.*, 2002; Haussler and Becker, 2008).

A mutation in any quorum sensing system leads to attenuated virulence of *P. aeruginosa*. Interestingly, RhlR can compensate a *lasR* mutation by activating essential LasR controlled functions (Dekimpe and Deziel, 2009).

It is also described that quorum sensing signalling molecules have immuno modulating functions. Neutrophil apoptosis and cytokine release is reported to be induced by odDHL (Smith *et al.*, 2001). Quorum sensing signalling molecules probably allow the cross talk between different bacterial species (Federle and Bassler, 2003).

1.2.3 Adaptation strategies and virulence of *Pseudomonas aeruginosa* during chronic cystic fibrosis infection

Adaptive evolution due to genetic variation seems to be one of the key aspects of *P. aeruginosa* survival in the challenging environment of the CF lung during chronic infection (D'Argenio *et al.*, 2007; Mena *et al.*, 2008; Smith *et al.*, 2006).

Host immune response and reoccurring antibiotic treatment create selective pressure. Oxygen limitation and the microaerobic to anaerobic conditions in the CF lung are also important factors shaping the *P. aeruginosa* phenotype (Alvarez-Ortega and Harwood, 2007; Worlitzsch *et al.*, 2002). This environment leads to the known phenotypes of mucoidy and the formation of microcolonies and biofilms (Boucher *et al.*, 1997; Costerton *et al.*, 1999; Govan and Deretic, 1996; Lam *et al.*, 1980).

Clinically relevant phenotypic changes are detectable, as *P. aeruginosa* colonizes the nutrient rich mucus of the CF airways, such as colony variation (von Gotz *et al.*, 2004), loss of motility (Amiel *et al.*, 2010; Mahenthiralingam *et al.*, 1994), modification of LPS (Hancock *et al.*, 1983), loss of quorum sensing (D'Argenio *et al.*, 2007; Smith *et al.*, 2006), reduced virulence (Luzar and Montie, 1985) and increased antibiotic resistance (Ciofu *et al.*, 2001; Wiegand *et al.*, 2008) (Fig. 3). In addition, it was shown that the mutator phenotype is widely spread among *P. aeruginosa* CF isolates and very important for adaptation (Ciofu *et al.*, 2005; Hogardt *et al.*, 2006; Oliver *et al.*, 2000).

Mutator phenotypes arise due to mutations in the methyl-directed DNA mismatch repair (MMR) and the guanine 8-oxo-2-deoxyguanosine (GO) system (Ciofu *et al.*, 2010). Most of the frequently isolated mutators in CF carry mutations in *mutS* and *mutL* genes belonging to the MMR type. Strong mutators display a mutation frequency of at least 100 to 1000-fold increased. Defects in the GO system genes result in a weak mutator phenotype ($<2 \times 10^{-7}$ and $\geq 2 \times 10^{-8}$ for

rifampicin) (Ciofu *et al.*, 2010). The GO system is involved in the repair of DNA damaged by oxidation. It is encoded by *mutT*, *mutY* and *mutM* genes.

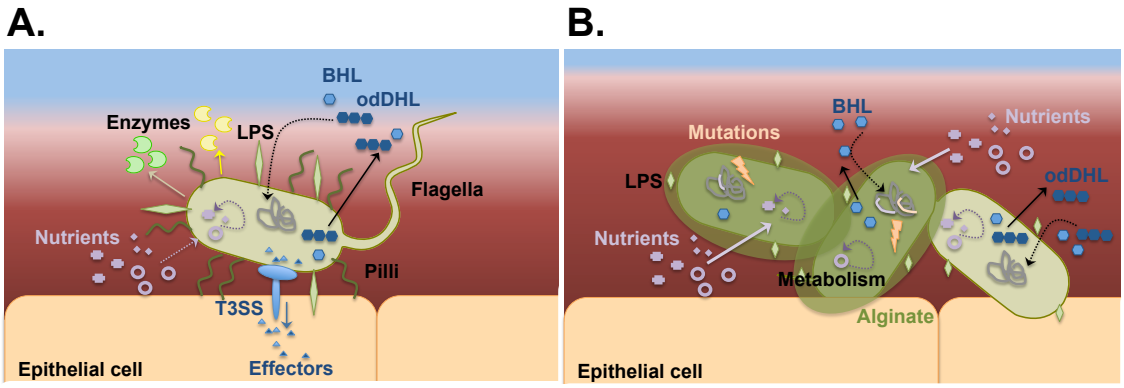


Fig. 3: Phenotypic adaptation of *Pseudomonas aeruginosa* during acute and chronic cystic fibrosis infection

(A) During acute infection *P. aeruginosa* strains are motile by flagella. Many strains express type IV pili for surface attachment. Quorum sensing molecules odDHL and BHL are produced and secreted. Upon threshold achievement, regulation of gene expression of certain genes is taking place. Type three secretion system (T3SS) is used for the translocation of effector molecules into the host cells. Several enzymes such as e.g. phospholipase C, elastase LasB, staphylolytic protease LasA and alkaline protease are secreted into the mucus to degrade nutrients. Iron scavenger as the siderophores pyoverdin and pyochelin are secreted.

(B) During chronic infection *P. aeruginosa* strains are often non-motile. Overproduction of alginate and loss of quorum sensing are the characteristics of transition to chronic CF infection. The *P. aeruginosa lasR* mutants are frequently isolated, which do not produce odDHL. Modifications of LPS are also detected. Mutations in the genome are also very common and the mutator phenotype is frequently observed. Few strains require certain nutrients for growth e.g. amino acids. Bacteria attach to each other and form microcolonies and biofilms. During growth in the microaerobic to anaerobic environment arginine fermentation or anaerobic nitrate respiration are used. Very different phenotypes are isolated form the airways of CF patients. The mucus is rich in amino acids of degraded proteins, glycoproteins, DNA, phospholipids, electrolytes, cellular debris from neutrophils and bacteria. Nitrate concentrations up to 700 µM were detected in the CF sputum.

Loss of function mutations in the genes of *mucA* and *lasR* are frequently observed. The mutations result in either mucoidy or the partial loss of quorum sensing. Both are evidence for the transition to chronic *P. aeruginosa* infection (Fig. 3 B).

The excessive production of alginate, which is the main component of the biofilm matrix, results in reduced oxygen diffusion to the bacteria and causes a switch to microaerobic and anaerobic metabolism (Boucher *et al.*, 1997). The diffusion barrier protects the bacteria also from antibiotics (Ratjen and Doring, 2003), oxygen radicals (Mathee *et al.*, 1999) and prevents phagocytosis (Schwarzmann and Boring, 1971; Worlitzsch *et al.*, 2002). Therefore, the loss of function mutation of the *mucA* gene leads to diminished possibility of *P. aeruginosa* eradication by either antibiotics or host immune system. Interestingly, during the end-stage of CF the

domination of the mucoid phenotype is reverted to the non-mucoid phenotype. This is indicating a change of selective pressure (Bragonzi *et al.*, 2009).

The loss-of-function mutation of *lasR* gene results in reduced expression of virulence factor genes and allows on the contrary more efficient utilization of certain carbon sources such as phenylalanine and other aromatic and branched-chain amino acids (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2010; Sandoz *et al.*, 2007). This is probably due to the upregulation of the transcriptional metabolism regulator *cbrB* gene (D'Argenio *et al.*, 2007). Hoffmann *et al.* (2010) detected increased utilization of nitrate and nitrite but decreased utilization of oxygen of the *lasR* mutants. Resistance towards oxidative stress, including antibiotics such as tobramycin and ciprofloxacin was also increased in *lasR* mutants. Moreover, *lasR* mutants are frequently detected in the sputum of CF patients without co-isolation of wild type (wt) strains (D'Argenio *et al.*, 2007; Smith *et al.*, 2006).

Moreover, pyocyanin-negative strains were isolated. Hogardt and Heesemann (2013) assumed that this might reduce the auto-oxidation stress of *P. aeruginosa* and might lead to reduced recognition by the host immune system.

Previous studies investigated the influence of nutrient availability and its direct influence on bacterial metabolism, on growth and virulence factor production (Palmer *et al.*, 2007a; Palmer *et al.*, 2005; Son *et al.*, 2007; Sriramulu *et al.*, 2005). In addition, it was frequently observed that some *P. aeruginosa* strains isolated from the CF lung were unable to synthesise particular growth factors (Taylor *et al.*, 1992). Strains were auxotrophic for certain nutrients. The most common source of auxotrophy was amino acids in *P. aeruginosa*. Most frequently methionine auxotrophic *P. aeruginosa* strains were isolated (Barth and Pitt, 1995; Barth and Pitt, 1996; Barth *et al.*, 1998; Thomas *et al.*, 2000). Other less frequently observed auxotrophies were identified as auxotrophies for the amino acids leucine, arginine and for both isoleucine and valine (Barth and Pitt, 1995; Barth and Pitt, 1996; Taylor *et al.*, 1992).

Biofilm development has been shown to be influenced by carbon and nitrogen sources *in vitro* (Hoffman *et al.*, 2010; Klausen *et al.*, 2003; Shrout *et al.*, 2006; Sriramulu *et al.*, 2005). Furthermore, cell-to-cell signalling is also influenced by the nutrient availability (Palmer *et al.*, 2005; Shrout *et al.*, 2006; Wagner *et al.*, 2003).

Especially, 2-heptyl-3-hydroxyl-4-quinolone the PQS signal seems to be an important signalling molecule under CF conditions. Several studies suggest that the PQS production is increased due to the moiety of aromatic amino acids in the CF sputum (Palmer *et al.*, 2007a; Palmer *et al.*, 2005).

1.2.4 Iron acquisition and uptake by *Pseudomonas aeruginosa*

Iron is one of the important factors for *P. aeruginosa* to establish and maintain an infection. Therefore, acquisition and storage of iron are essential under iron limiting conditions such as in the environment or during infection. Iron concentrations range from < 0.1 µM in the soil to 10^{-9} µM in mammals (Vasil and Ochsner, 1999). The host tightly regulates iron availability by binding it to proteins such as ferritin, lactoferrin, transferrin or as heme to haemoglobin.

Typically bacteria e.g. *E. coli* need approximately 0.3 -1.8 µM iron for optimal growth (Braun and Killmann, 1999). Free iron is usually limited in an aerobic environment, since it is highly

insoluble as Fe^{3+} but also harmful hydroxyl radicals can be easily generated via Fenton-type reactions (Miller and Britigan, 1997). Iron acquisition is essential for every bacterium, since especially redox-dependent enzymes need iron to be functional. In order to circumvent these limitations *P. aeruginosa* possesses a variety of different mechanisms, ranging from siderophore production, over heme uptake to extracellular toxin generation. Exotoxin A is inhibiting protein biosynthesis of the eukaryotic cell by ADP-ribosylation of elongation factor-2 (Iglewski and Kabat, 1975). It is strongly induced by iron depletion (Vasil and Ochsner, 1999).
Several studies observed, that shortage of iron led to increased expression of iron acquisition genes, such as the genes of the siderophores pyoverdin and pyochelin (Oglesby *et al.*, 2008; Palma *et al.*, 2003; Vasil and Ochsner, 1999).
Iron acquisition, uptake and storage are directly and indirectly controlled by the global ferric uptake regulator Fur, extracytoplasmic function (ECF) sigma factors or other types of regulators such as two-component regulatory systems and AraC-type regulators in *P. aeruginosa*. At iron availability the Fur protein is loaded with Fe^{2+}. The ferrated Fur binds to the Fur box in the promoter region of genes e.g. necessary for iron uptake, and thereby preventing transcription (Oglesby *et al.*, 2008). Several microarray studies have been performed to investigate iron regulated genes and the influence of iron starvation (Ochsner *et al.*, 2002; Palma *et al.*, 2003).
Previous studies reported that PQS is able to chelate iron, besides being a signaling molecule and being necessary for the formation of outer membrane vesicles (Bredenbruch *et al.*, 2005; Mashburn and Whiteley, 2005; Mashburn-Warren *et al.*, 2009). Thereby leading to activation of iron acquisition genes and genes necessary for oxidative stress protection (Bredenbruch *et al.*, 2005). But it is also indicated that PQS itself does not function as a siderophore, but might simplify siderophore mediated uptake (Diggle *et al.*, 2007b).

1.2.5 Methionine metabolism of *Pseudomonas aeruginosa*

An essential part of bacterial metabolism is the methionine and S-adenosylmethionine (SAM) metabolism, which are inseparable linked to one another (Fig. 17). Methionine and ATP are converted by S-adenosylmethionine synthetase MetK to SAM in *P. aeruginosa*. SAM is the second most frequently used enzyme substrate after ATP (Fontecave *et al.*, 2004; Loenen, 2006). The majority of estimated 95 % of SAM is used for methylation reactions (Griffith, 1987). Methylation is one of the most ubiquitous chemical reactions in cellular metabolism (Stepkowski *et al.*, 2005). S-adenosylhomocysteine (SAH) is the product of these reactions and a potent inhibitor of transmethylation reactions and therefore efficiently converted to homocysteine (Hcy) and adenosine (Ado) by S-adenosylhomocysteine hydrolase SahH. The methylation of macromolecules and small molecules is dependent on the intracellular SAM / SAH equilibrium. Recently, the intracellular SAM and SAH level for the *E. coli* wild type strain MG1665 (OD_{600} of 1.62 ± 0.16) was determined with a ratio of 300:1 with 0.4 mM and 1.3 µM, respectively (Halliday *et al.*, 2010). Hcy can also be generated by MetZ from O-succinyl-homoserine. The last step of methionine biosynthesis is the conversion of Hcy via either MetE or MetH to methionine. MetH is a cobalamin dependent and MetE a cobalamin independent methionine synthase. Both enzymes use the cofactor N^5-methyl-tetrahydrofolate (Me-THF) and N^5-methyl-

tetrahydropteroyltriglutamate (Me-THP) as methyl-group donor, respectively. Methionine can then be directly incorporated into biosynthesis or it can be converted to SAM as described.

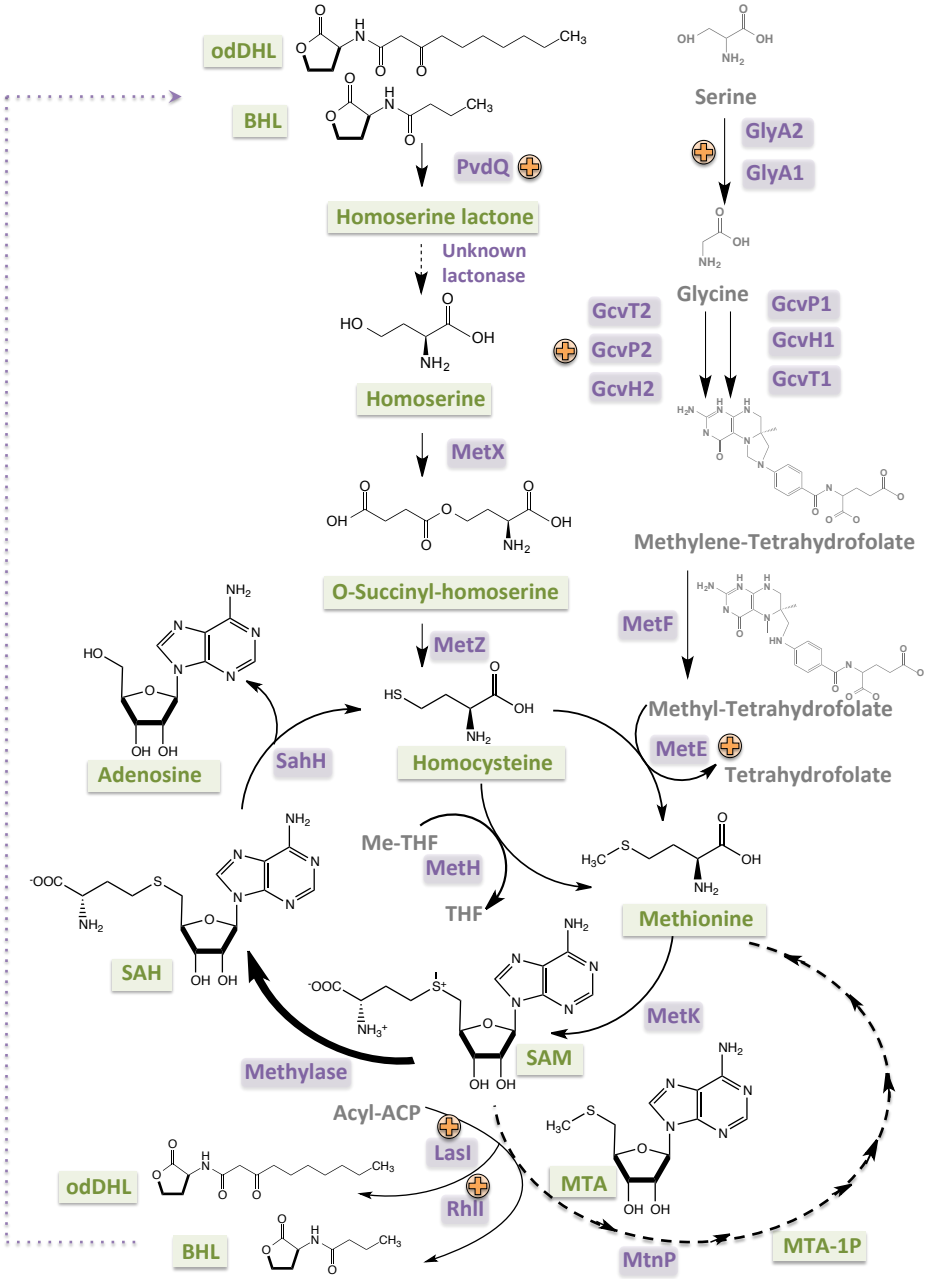


Fig. 4: Methionine metabolism and SAM recycling in *Pseudomonas aeruginosa*

Methionine and ATP are converted by S-adenosylmethionine synthetase MetK into SAM, phosphate and diphosphate. SAM is used for various metabolic reactions in the cell. If SAM is used for methylation the remaining SAH is hydrolysed by SahH into Ado and Hcy. Hcy can be generated out of O-succinyl-homoserine by MetZ. Hcy can be converted by either cobalamin-independent methionine synthase MetE or cobalamin-dependent methionine synthase MetH back into methionine. Both enzymes use the cofactor N^5-methyl-tetrahydrofolate (Me-THF) and N^5-methyl-tetrahydropteroyltriglutamate (Me-THP) as methyl-group donor, respectively (only THF is shown). MetF converts N^5,N^{10}-methylene-tetrahydrofolate to Me-THF or N^5,N^{10}-methylene-tetrahydropteroyltriglutamate to Me-THP. GlyA1 and GlyA2 are serine hydroxymethyl transferases, which convert serine into glycine and thereby produce N^5,N^{10}-methylene-tetrahydrofolate from THF.

The GcvT2H2P2 glycine cleavage system produces also N^5,N^{10}-methylene-tetrahydrofolate from THF and glycine.
If SAM is used for other reactions e.g. by AHL synthase or as source of an amino propyl group for the generation of spermidine these reactions yield 5´-methylthioadenosine (MTA). MTA is recycled by 5´-methylthioadenosine phosphorylase MtnP and by several other enzymes to methionine. N-butyryl homoserine lactone (BHL) is synthesized by RhlI and N-3-oxododecanoyl homoserine lactone (odDHL) by LasI. Several genes encoding for methionine metabolism enzymes are positively regulated by quorum sensing (indicated by orange plus). Adapted from (Fontecave *et al.*, 2004; Heurlier *et al.*, 2006; Parveen and Cornell, 2011; Sekowska *et al.*, 2004).

Approximately 2-5% of cellular SAM is used for decarboxylation reactions e.g. for the production of polyamines, for quorum sensing molecule biosynthesis for N-acylhomoserine lactones (AHLs) or as source of 5´deoxyadenosyl radicals, which are necessary for the production of vitamins e.g. biotin and lipoate etc. (Griffith, 1987; Parveen and Cornell, 2011). These reactions yield 5´-methylthioadenosine (MTA) or 5´deoxyadenosine (5´dAdo), respectively.
Guan *et al.* (2011) propose that MTA is converted into 5´-methylthioinosine (MTI), which is further metabolised into hypoxanthine and 5´-methylthioribose-1-phosphate (MTR-1-P). MTR-1-P is further recycled into methionine by the gene products of *mtnA*, *mtnB*, *mtnC*, *mtnD* and *tyrB* (Heurlier *et al.*, 2006).
P. aeruginosa is one of the few bacteria, which possesses a SAH hydrolase as well as a MTA phosphorylase (MtnP, EC 2.4.2.28) for recycling of SAH and MTA, and probably for 5´dAdo (Challand *et al.*, 2010; Challand *et al.*, 2009; Choi-Rhee and Cronan, 2005). The narrow substrate specificity of both enzymes for the recycling of either SAH or MTA seems to be an advantage for survival of *P. aeruginosa* under the varying environmental conditions (Stepkowski *et al.*, 2005). This is very similar to what can be found in eukaryotes, including mammals, besides it is found several Archaea and other bacterial species with relatively large genomes (>6 Mb) (Stepkowski *et al.*, 2005).
Interestingly, AHL biosynthesis is connected to the methionine and SAM metabolism. AHLs are synthesized from SAM and an intermediate of fatty acid biosynthesis by the AHL synthases of the LuxI family (Heurlier *et al.*, 2006). N-butyryl homoserine lactone (BHL) is synthesized by RhlI and N-3-oxododecanoyl homoserine lactone (odDHL) by LasI. BHL can diffuse freely (Wagner *et al.*, 2003; Williams and Camara, 2009). odDHL can also diffuse but its transport is increased by *mexAB-oprM* and probably other efflux pumps.
Interestingly, several genes of methionine and SAM metabolism are described to be under positive quorum sensing control such as the genes encoding for the cleavage system *gcvT2H2P2*, *glyA1* hydroxy-methyltransferase, *pvdQ* and *metE* (Schuster *et al.*, 2003; Wagner *et al.*, 2003).

1.2.6 Riboswitches in methionine metabolism of *Pseudomonas aeruginosa*

Other regulating factors seem to have an important impact on methionine and SAM metabolism genes and might be potentially targeted by drugs. Riboswitches are mRNA structures that

regulate gene expression (Blount and Breaker, 2006). Blount and Breaker (2006) proposed that metabolite sensing RNAs (riboswitches) could also be potential targets.

For several bacterial species SAM and SAH dependent riboswitches were shown or were predicted in the mRNAs of the genes involved in the methionine and SAM metabolism. For *Pseudomonas syringae* a SAH dependent riboswitch in the mRNA of the *sahH* operon was described (Wang *et al.*, 2008). Wang *et al.* (2008) proposed that SAH binding activates gene expression. Furthermore, 15 SAH RNA motifs were identified in the Pseudomonadales (Wang *et al.*, 2008). Recently, a SAM-III box was identified in the 5´untranslated region (5´ UTR) of *metK* in *Enterococcus faecalis* (Smith *et al.*, 2010).

So far it is predicted and partly verified for some organisms that the genes of S-adenosylhomocysteine hydrolase (*sahH*), cobalamin-dependent methionine synthase (*metH*) and methylenetetrahydrofolate reductase (*metF*) are possessing possible SAH elements in the 5´ UTR (Wang *et al.*, 2008; Weinberg *et al.*, 2007).

1.2.7 Potential drug targets in *Pseudomonas aeruginosa*

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine analogue. It is structurally very similar to adenosine (Ado), e.g. inter alia the product of the hydrolysis of SAH into Hcy and Ado (Fig. 5). Ribavirin is an approved drug against DNA and RNA viruses, e.g. for hepatitis C treatment in combination with interferon (Davis *et al.*, 1998). Recently, ribavirin's impact on *Trypanosmoma cruzi´s* S-adenosylhomocysteine hydrolase SAHH was successfully tested (Cai *et al.*, 2010; Cai *et al.*, 2007). Ribavirin resulted in total inhibition of the enzyme in an *in vitro* assay. Furthermore, looking for antimicrobial activity in non-antibiotic drugs Kruszewska *et al.* (2002) tested ribavirin successfully in their study. In addition Kruszewska *et al.* showed an effect against *P. aeruginosa*.

S-Adenosylhomocysteine hydrolase (SahH, EC 3.3.1.1) has been identified as a potential drug target in one of the essential metabolic pathways the recycling of SAM. Due to narrow substrate specificity SahH is predicted to be an excellent drug target.

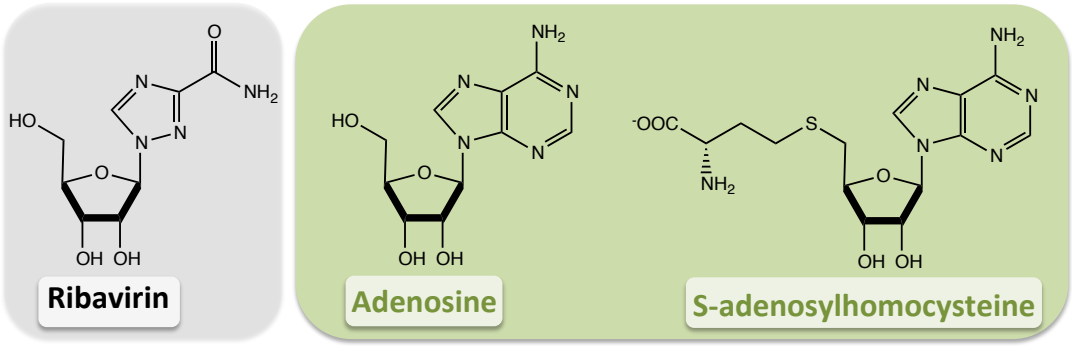


Fig. 5: Chemical structure of ribavirin, adenosine and S-adenosylhomocysteine

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is as a purine analogue structurally very similar to adenosine. Adenosine is e.g. inter alia the product of the hydrolysis of S-adenosylhomocysteine (SAH) into homocysteine (Hcy) and adenosine (Ado).

1.3 Aim of this study

The aim of this study is divided into three parts. The first part focuses on the *in vivo* situation during the chronic CF airway infection by *Pseudomonas* aeruginosa. Still very little is known about the impact of the CF airways and its nutrient availability as surrounding growth environment on the infecting *P. aeruginosa* strains.

Transcriptional profiling should be employed to inverstigate the difference in gene expression of *P. aeruginosa* grown under *ex vivo* CF airway conditions or under simulated respiratory tract conditions in an existing artificial sputum medium (ASM) by Sriramulu *et al.* (2005). Besides understanding the underlying adaptation strategies, insights into regulatory networks, the environmental responses and the identification of crucial factors, it was the goal to modify the existing ASM to simulate the *ex vivo* conditions more precisely.

The second part deals with the question of the high frequency of methionine auxotrophic *P. aeruginosa* strains among the CF isolates. The main goal was the identification of the genetic cause of methionine auxotrophy. A representative number of isolates should be analysed. The influence of methionine auxotrophy on virulence factor production should be investigated.

To understand the underlying adaptation strategies, several methionine metabolism gene deletion mutants in PAO1 should be constructed. A phenotypic characterisation of the methionine auxotrophic strains and PAO1 methionine metabolism deletion mutants should be performed in order to determine the advantages of methionine auxotrophy

The third part of the study reports about both, a new antibiotic target and about an already known viral drug effective against *P. aeruginosa*, since multidrug resistance is spreading new targets and substances are necessary.

The possibility of ribavirin usage against *P. aeruginosa* PAO1 should be elucidated. SahH as potential drug target in *P. aeruginosa* should be investigated and the mode of action of ribavirin should be analysed. Therefore, it would also be of importance to investigate the influence of SahH and MtnP on bacterial phenotype. In addition, the presence of new regulatory elements such as riboswitches in the 5´ UTR of methionine metabolism genes in *P. aeruginosa* should be analysed. An *in vitro* assay should be established to analyse the impact of the inhibitor ribavirin on the activity of enzyme SahH of *P. aeruginosa* PAO1.

2 Iron limitation - the abiotic factor dominating the cystic fibrosis lung infection

2.1 Introduction

Still very little is known about the *in vivo* situation during the chronic cystic fibrosis (CF) airway infection by *Pseudomonas aeruginosa.* Although it has been extensively studied for years.

CF is an autosomal hereditary disease (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). Besides the mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Hauser *et al.*, 2011; Lyczak *et al.*, 2002) a variety of innate immune system dysfunctions have been reported, which all together promote a chronic bacterial lung infection (Doring and Gulbins, 2009). Once a chronic *P. aeruginosa* airway infection is established, the infection is nearly impossible to cure and often associated with the decline of lung function and a reduced life expectancy (Doring and Gulbins, 2009; Hauser *et al.*, 2011).

P. aeruginosa is a ubiquitously spread versatile Gram-negative bacterium (Stover *et al.*, 2000). Moreover, *P. aeruginosa* is known as opportunistic and nosocomial pathogen, especially of immunocompromised patients, due to its intrinsic resistances (Hancock, 1998), besides its prevalence in CF. But the question remains, how do the CF airways and its nutrient availability as surrounding growth environment affect the *P. aeruginosa* infection?

Clinically relevant phenotypic changes are detectable, as *P. aeruginosa* colonizes the nutrient rich mucus of the CF airways, such as colony variation, loss of motility, modification of LPS and auxotrophy (Amiel *et al.*, 2010; Barth and Pitt, 1995; Barth and Pitt, 1996; Hancock *et al.*, 1983; von Gotz *et al.*, 2004). This special environment leads to the known phenotypes of mucoidy and the formation of microcolonies and biofilms (Boucher *et al.*, 1997; Govan and Deretic, 1996; Lam *et al.*, 1980). Oxygen limitation and the microaerobic to anaerobic conditions in the CF lung are also important factors shaping the *P. aeruginosa* phenotype (Alvarez-Ortega and Harwood, 2007; Worlitzsch *et al.*, 2002).

Several studies have investigated the influence of nutrient availability and its direct influence on bacterial metabolism and the resulting influence on growth behaviour and virulence factor production (Palmer *et al.*, 2007a; Palmer *et al.*, 2005; Son *et al.*, 2007; Sriramulu *et al.*, 2005).

Biofilm development has been shown to be influenced by carbon and nitrogen sources *in vitro* (Hoffman *et al.*, 2010; Klausen *et al.*, 2003; Shrout *et al.*, 2006; Sriramulu *et al.*, 2005). Furthermore cell-to-cell signalling (quorum sensing) is also affected by the nutrient availability (Palmer *et al.*, 2005; Shrout *et al.*, 2006; Wagner *et al.*, 2003). Especially, 2-heptyl-3-hydroxyl-4-quinolone the Pseudomonas quinolone signal (PQS), seems to be an important signalling molecule under CF conditions. Several studies suggest that the PQS production is increased due to the moiety of aromatic amino acids in the CF sputum (Palmer *et al.*, 2007a; Palmer *et al.*, 2005). Besides, being a signal molecule, PQS is also necessary for the formation of outer membrane vesicles (Mashburn and Whiteley, 2005; Mashburn-Warren *et al.*, 2009). Other studies report also that PQS is able to chelate iron (Bredenbruch *et al.*, 2005) and thereby leading to the activation of iron acquisition genes and genes necessary for oxidative stress protection. But it is also indicated that PQS itself does not function as a siderophore, but might simplify siderophore mediated uptake (Diggle *et al.*, 2007b).

Iron is one of the important factors, not only for *P. aeruginosa,* to establish and maintain an infection. Acquisition and storage of iron are essential under iron limiting conditions such as in the environment or during infection. Iron concentrations range from < 0.1 µM in the soil to 10^{-9} µM in mammals (Vasil and Ochsner, 1999). The host tightly regulates iron availability by binding it to proteins such as ferritin, lactoferrin, transferrin or as heme to haemoglobin.

Typically bacteria e.g. *Escherichia coli* need approximately 0.3 -1.8 µM iron for optimal growth (Braun and Killmann, 1999). Free iron is usually limited in an aerobic environment, since it is highly insoluble as Fe^{3+}, but also harmful hydroxyl radicals can be easily generated via Fenton-type reactions (Miller and Britigan, 1997). Iron acquisition is essential for every bacterium. Especially redox-dependent enzymes need iron to be functional. In order to circumvent these limitations *P. aeruginosa* possesses a variety of different mechanisms, ranging from siderophore production, over heme uptake to extracellular toxin generation. Exotoxin A is inhibiting protein biosynthesis of the eukaryotic cell by ADP-ribosylation of elongation factor-2 (Iglewski and Kabat, 1975). It is strongly induced by iron depletion (Vasil and Ochsner, 1999). Several studies observed, that shortage of iron led to increased expression of iron acquisition genes, such as siderophores pyoverdin and pyochelin biosynthesis genes (Oglesby *et al.*, 2008; Palma *et al.*, 2003; Vasil and Ochsner, 1999).

Iron acquisition, uptake and storage are directly or indirectly controlled by the global ferric uptake regulator Fur, extracytoplasmic function (ECF) sigma factors or other types of regulators such as two-component regulatory systems and AraC-type regulators in *P. aeruginosa*. At iron availability the Fur protein is loaded with Fe^{2+}. The ferrated Fur binds to the Fur box in the promoter region of genes e.g. necessary for iron uptake, and thereby preventing transcription (Oglesby *et al.*, 2008). Several microarray studies have been performed to investigate iron regulated genes and the influence of iron starvation (Ochsner *et al.*, 2002; Palma *et al.*, 2003).

CF airways colonizing *P. aeruginosa* are confronted with a highly selective and heterogeneous environment, which is characterised by various stress conditions such as antibiotics, host immune response and oxidative and osmotic stress (Folkesson *et al.*, 2012).

Transcriptional profiling of *P. aeruginosa* under CF airway conditions in *ex vivo* sputum samples and under simulated respiratory tract conditions in the existing artificial sputum medium (ASM) by Sriramulu *et al.* (2005) was used. Besides understanding the underlying adaptation strategies, insights into regulatory networks, the environmental responses and the identification of crucial factors, it is the goal to modify the existing ASM to simulate the *in vivo* conditions precisely.

2.2 Results & Discussion

2.2.1 *Pseudomonas aeruginosa* isolates from cystic fibrosis patients

In order to understand and identify the important factors, which allow the chronic colonization of the cystic fibrosis airways by *P. aeruginosa*, the colonizing strains of two independent CF patients were isolated by Dr. Piotr Bielecki. Furthermore, the RNA obtained from the patient's sputum using microarrays designed for *P. aeruginosa* PAO1 genome analysis were examined (Bielecki *et al.*, 2013). Besides understanding the underlying adaptation strategies and the identification of crucial factors, it was the goal to compare the growth conditions in the existing artificial sputum medium (ASM) by Sriramulu *et al.* (2005) to *ex vivo* sputum samples of *P. aeruginosa* strains directly isolated from CF-patients' airways.

The clinical strains were tested for clonal variability using binary arrays based on o single-nucleotide polymorphisms (SNPs) genotypes (Wiehlmann *et al.*, 2007). The isolate CFCZ (patient A) and the isolates CF-1 to CF-4 (patient B) belong to clonally distinct groups. CFCZ is grouped into the clonal group E59A. A clone form this group is already present in the MHH-collection. The previous isolate was obtained in 1985 from a CF patient in Hanover (Germany) (Wiehlmann *et al.*, 2007).

The isolates CF-1, CF-2, CF-3 and CF-4 belong to the clonal group 6C2A, which are representatives of the clone P. All four clones show the same SNP pattern in the array. However, the colonies appear morphologically different. The clone P is also present in the MHH collection and the MHH isolates were isolated form CF patients in various places in Germany during the last 17 years.

2.2.2 Iron limitation is stronger *ex vivo* than in artificial sputum medium

The transcriptional profiles of *ex vivo* sputum samples of the strains CFCZ and CF-1 to CF-4 were compared to those determined in ASM. The transcriptional profiles for each strain and each condition were performed in duplicate. The five clinical *P. aeruginosa* strains used in this study were considered as one data set for the purpose of analysis, thereby creating a more general picture of *P. aeruginosa* characteristics, rather than strain specific features. Genes that showed significant difference in expression (percentage of false-positives pfp < 0.05) were further analysed and functionally classified according to "Pseudomonas Genome Database" (Fig. 6) (Winsor *et al.*, 2011).

404 genes were regulated differently during growth in ASM compared to *ex vivo* sputum samples. That means that 7.3 % of the 5570 predicted open reading frames (ORFs) (Stover *et al.*, 2000) were expressed differently in ASM compared to *ex vivo* sputum samples. 205 genes were upregulated in ASM compared to *ex vivo*. 199 genes were downregulated. Significant induction was seen for genes classified as carbon compound catabolism genes. The same was observed for energy metabolism and putative enzymes genes (Fig. 6). Genes concerning adaptation and protection were downregulated, just as membrane protein genes and genes regarding the transport of small molecules.

A detailed look into the data revealed that the majority of genes regulated, were indeed regulated by iron. Genes were downregulated in ASM, whereas they were clearly upregulated under *ex vivo* conditions (Table 3 or Appendix Table 30), especially genes of transcriptional

regulators e.g. *pvdS*, *vreA*, *femI*, *foxI*, *pchR* and *pfeR* (Table 1 and Fig. 11), which are repressed by Fur in the presence of Fe^{2+}, were downregulated.

Table 1: Fold change of gene expression of selected iron regulated transcriptional regulators in ASM compared to *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4 grown in artificial sputum medium ASM (A) in comparison with *ex vivo* (EV) sputum samples.

Gene ID[a]	Gene name[a]	Function[a]	FC A / EV
PA0674	*vreA*	VreA	-21,4
PA1300		probable sigma-70 factor, ECF subfamily	-42,2
PA1912	*femI*	ECF sigma factor, FemI	-11,1
PA2426	*pvdS*	sigma factor PvdS	-71,9
PA2468	*foxI*	ECF sigma factor FoxI	-7,9
PA3899		probable sigma-70 factor, ECF subfamily	-20,7
PA4227	*pchR*	transcriptional regulator PchR	-70,9
PA4896		probable sigma-70 factor, ECF subfamily	-21,5
PA2686	*pfeR*	two-component response regulator PfeR	-5,1

[a] Gene ID, gene name, function and PseudoCAP Function Class (PCFC) are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

In total 77 genes, which are involved in iron uptake and storage, were downregulated. This indicates that one fifth of all regulated genes are iron regulated. Iron starvation is one of the important factors in the airways of CF patients, whereas the effect of iron limitation is not that strong in ASM. Although ASM contains diethylene triamine pentaacetic acid (DTPA), as chelating substance in order to sequester free metal ions such as free iron.

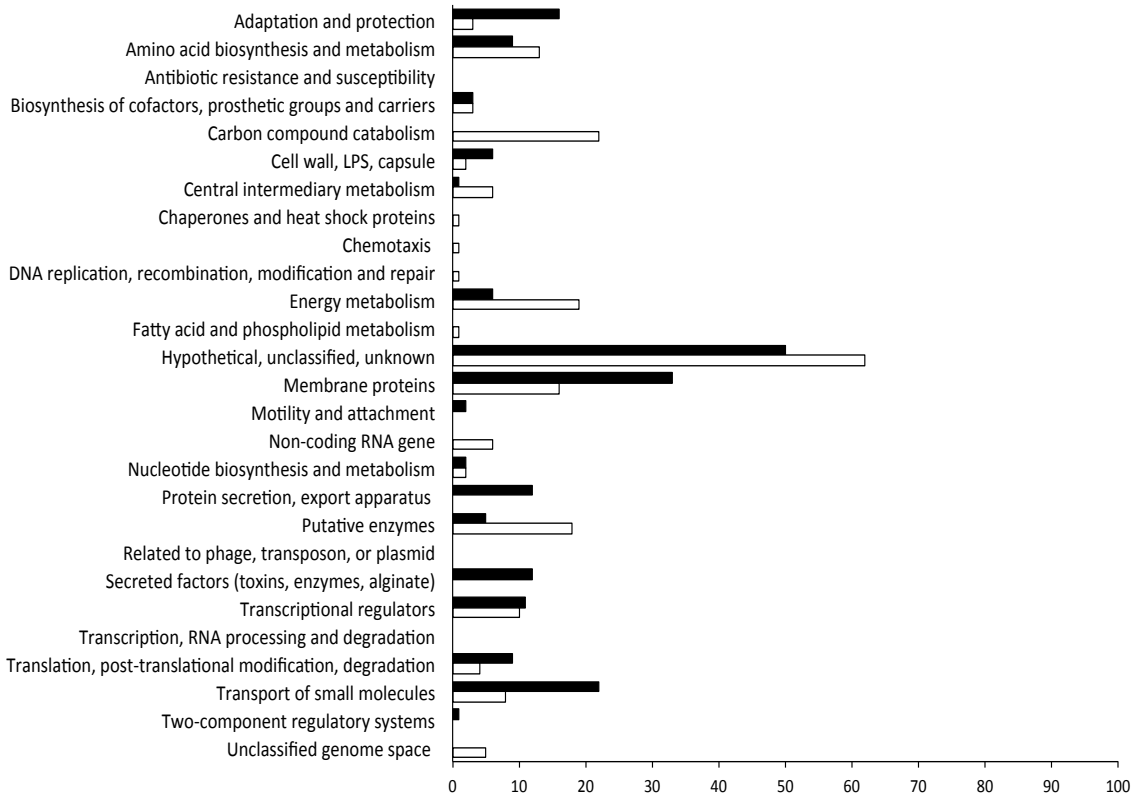


Fig. 6: Functional classification of genes differentially regulated during growth in ASM compared to *ex vivo* sputum samples of the the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4

Differentially regulated genes (pfp < 0.05) are classified according to "Pseudomonas Genome Database" (Winsor *et al.*, 2011). Black bars represent downregulated genes, whereas white bars represent upregulated genes in ASM compared to *ex vivo* conditions.

2.2.3 Quantitative real-time polymerase chain reaction to establish iron limitation in ASM

Since iron limitation is one of the obstacles *P. aeruginosa* has to overcome in order to establish and maintain an infection in the CF airways, the approach focused on selected genes necessary for iron acquisition and uptake as marker genes. Therefore, different compounds were tested in ASM to increase the iron limitation response. ASM contains the iron chelator diethylene triamine pentaacetic acid (DTPA). But previously conducted experiments indicated that *P. aeruginosa* is able to metabolise DTPA. Therefore, DTPA was not further analysed as possible iron chelator.

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to monitor the degree of iron limitation in modified ASMs. On the one hand naturally occurring iron chelating compounds such as lactoferrin and conalbumin were applied and on the other hand a chemically generated compound the resin Chelex with a high preference for iron was applied. Iron regulated genes for further investigation were selected, which were clearly downregulated in ASM compared to *ex vivo* samples (Table 2). These genes are involved in the biosynthesis and uptake of the siderophores pyoverdine (*pvdA*, *pvdF*, *fpvA*) and pyochelin (*fptA*, *pchG*). The expression of the *pvdS* gene was monitored. The transcriptional regulator PvdS regulates the expression of pyoverdine biosynthesis and uptake genes, as well as the expression of the virulence factor exotoxin A and other genes (Vasil, 2007).

Table 2: Fold change of gene expression of selected iron regulated genes chosen for further investigation by qRT-PCR to establish iron limitation in modified ASM

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4 grown in artificial sputum medium ASM (A) in comparison with *ex vivo* (EV) sputum samples.

Gene ID[a]	Gene name[a]	Function[a]	PseudoCAP Function Class (PCFC)	FC A / EV
PA2386	*pvdA*	L-ornithine N5-oxygenase	Adaptation, Protection	-62,5
PA2396	*pvdF*	pyoverdine synthetase F	Secreted Factors; Adaptation, Protection	-18,8
PA2398	*fpvA*	ferripyoverdine receptor	Transport of small molecules	-101,0
PA2426	*pvdS*	sigma factor PvdS	Transcriptional regulators	-71,9
PA4221	*fptA*	Fe(III)-pyochelin o.m. receptor precursor	Transport of small molecules	-41,7
PA4224	*pchG*	pyochelin biosynthetic protein PchG	Transport of small molecules ; Membrane proteins	-76,3

[a] Gene ID, gene name, function and PseudoCAP Function Class (PCFC) are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

P. aeruginosa was grown for 24 h in the modified ASMs, the RNA was isolated, cDNA was generated to perform qRT-PCR and the degree of iron limitation was analysed by monitoring the above-mentioned genes. The selected genes were compared under each condition to unmodified ASM and the fold difference was determined. All compounds selected for the modification of ASM induced expression of the investigated genes (Fig. 7). Thereby, it was

confirmed that lactoferrin, conalbumin and Chelex are capable of inducing iron limitation in ASM.

0.5 mg/ml and 1 mg/ml of iron-unsaturated lactoferrin were used. This reflects the naturally occuring lactoferrin concentrations within the airway respiratory secretions of 0.1 - 1 mg/ml (Harbitz *et al.*, 1984; Singh *et al.*, 2002).

Furthermore, the effect of 3 mg/ml and 4 mg/ml iron-unsaturated conalbumin in ASM was investigated. Conalbumin is also a lactoferrin-like host defence protein from chicken eggs (Singh *et al.*, 2002). 3 mg/ml and 4 mg/ml conalbumin led to induction of all investigated genes. For nearly all genes 3 mg/ml resulted in the same activation as 4 mg/ml, except for *fptA*. Conalbumin caused a stronger induction of investigated genes than lactoferrin. 0.1 mg/ml of the resin Chelex induced less iron limitation than 3 mg/ml and 4 mg/ml conalbumin, but more than 0.5 mg/ml and 1 mg/ml lactoferrin.

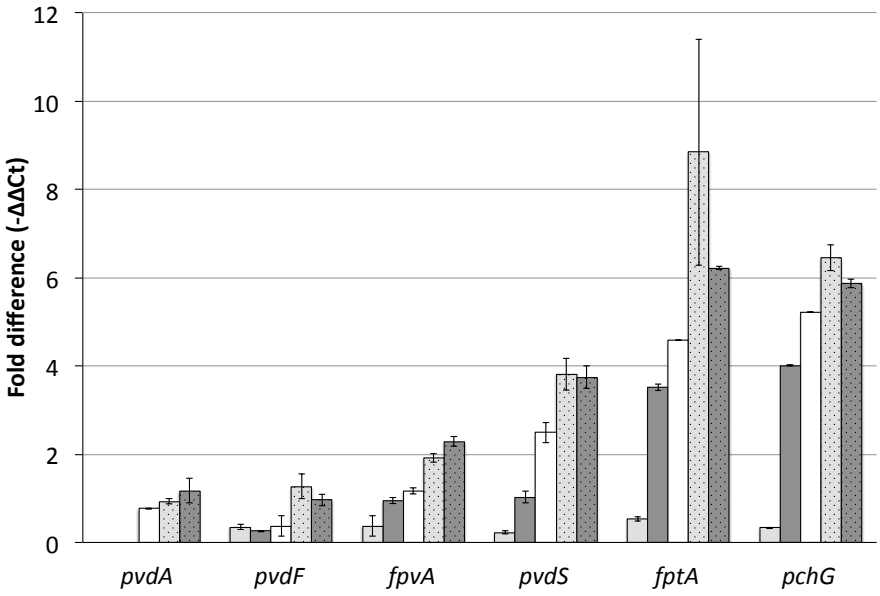


Fig. 7: Fold difference in gene expression of *Pseudomonas aeruginosa* iron limitation marker genes in differently supplemented artificial sputum media

In order to establish iron limitation in ASM it was modified by addition of 0.5 mg/ml lactoferrin (light grey bars), 1 mg/ml lactoferrin (dark grey bars), 0.1 g/ml Chelex (white bars), 3 mg/ml conalbumin (light grey dotted bars) or 4 mg/ml conalbumin (dark grey dotted bars), respectively. The genes *pvdA*, *pvdF*, *fpvA*, *pvdS*, *fptA* and *pchG* were monitored via qRT PCR for induction in response of iron starvation. PAO1 was grown for 24 h in modified ASMs. RNA was prepared and cDNA generated. Unmodified ASM was used as reference condition and *acpP* as reference gene for normalisation. The standard deviation of the mean is shown. For 0.5 mg/ml and 1 mg/ml lactoferrin *pvdA* was not determined. The strongest induction was observed for ASM-Chelex and ASM-Conalbumin.

Roger *et al.* (2004) reported that lactoferrin inhibited the biofilm formation of *P. aeruginosa*. Singh *et al.* (2002) confirmed the same effect for conalbumin. They assumed that iron chelation led to increased surface motility by twitching motility and thereby inhibiting biofilm formation (Singh *et al.*, 2002). Other studies report that lactoferrin is released from neutrophil granules in inflamed areas (Rogan *et al.*, 2006; Rogan *et al.*, 2004). Furthermore, lactoferrin is suspected to

destabilise the outer membrane of Gram-negative bacteria. On the other hand it is predicted to possess anti-inflammatory activity (Rogan *et al.*, 2006). Besides it was shown that bronchoalveolar lavage (BAL) from CF patients with *P. aeruginosa* infection was depleted of lactoferrin (Britigan *et al.*, 1993; Rogan *et al.*, 2004). The studies suggested that lactoferrin degradation might be either due to proteases produced by *P. aeruginosa* or by host proteases, which are strongly induced during the constant inflammation during infection.

Therefore, it was decided to use ASM-Chelex (ACh) for further studies since iron starvation cannot be abolished during prolonged incubation by protease degradation. On the other hand, addition of a glycoprotein would add more nutrients to the medium besides induction of iron limitation.

2.2.4 Transcriptional profiles of *Pseudomonas aeruginosa* in ASM and ASM-Chelex compared to *ex vivo*

The *ex vivo* transcriptional profile of two different *P. aeruginosa* clonal groups isolated from two CF patients were compared with those determined in ASM, which simulates the respiratory tract conditions of the CF lung. It was discovered that the iron starvation is one of the major factors *in vivo*, but not that strong in ASM. Therefore, the medium was modified by adding 0.1 mg/ml of the resin Chelex to ASM and obtained ASM-Chelex. In total 633 genes were differently regulated under these three investigated conditions *ex vivo*, ASM and ASM-Chelex.

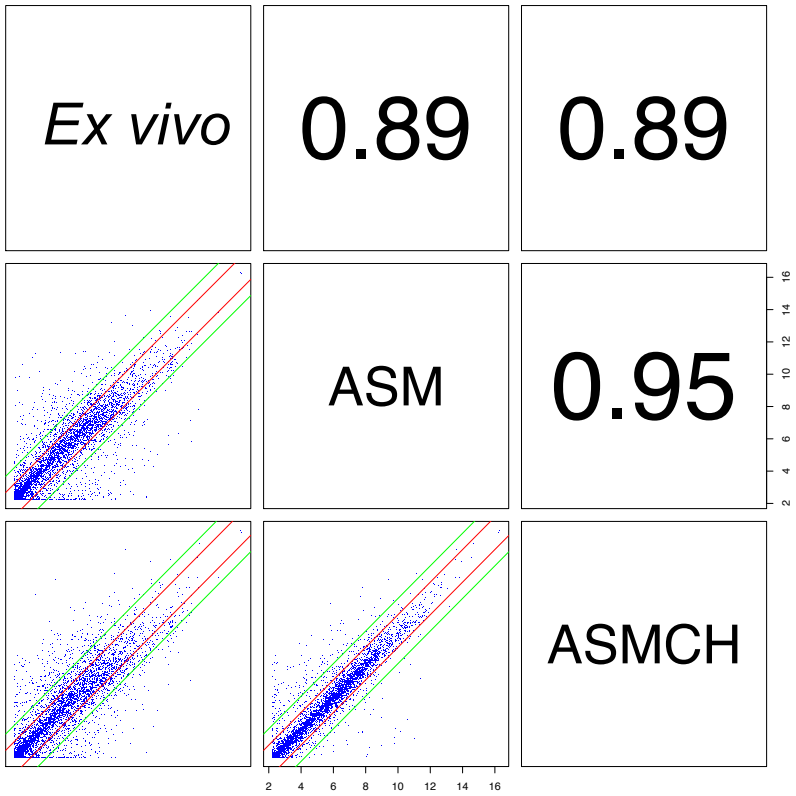


Fig. 8: Scatter plots and correlation values between the growth conditions of *ex vivo,* ASM and ASM-Chelex of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4

The scatter plot is a comparison of the regulation of all genes under the investigated conditions. Each condition represents four microarray chips, two of CFCZ and two of the strains CF-1 - 4. This study shows the three conditions *ex vivo*, ASM and ASM-Chelex (ASMCH) in total 12 microarrays are displayed.

The similarity in scatter plots and the very high correlation values of 0.89 for both comparisons demonstrate how well ASM and ASM-Chelex mimic the *ex vivo* environment of the CF airways (Fig. 8). The resulting high correlation value for both ASM and ASM-Chelex might be due to still very similar gene regulation in both media compared to the *ex vivo* conditions.

2.2.5 Transcriptional profile of ASM-Chelex vs. *ex vivo* sputum samples

The transcriptome of the growth conditions in ASM-Chelex was compared to *ex vivo* sputum samples. Genes that showed significant difference in expression (percentage of false-positives pfp < 0.05) were further analysed and functionally classified according to "Pseudomonas Genome Database" (Fig. 9) (Winsor *et al.*, 2011).

468 genes were regulated differently during growth in ASM-Chelex compared to *ex vivo* sputum samples. Upregulated were 247 genes in ASM-Chelex compared to *ex vivo* sputum samples. 221 genes were downregulated. Significant induction was seen for genes classified as amino acid biosynthesis and metabolism genes. The same was observed for energy metabolism and putative enzyme genes. Genes concerning adaptation and protection were downregulated as well as translation, post-translational modification and degradation.

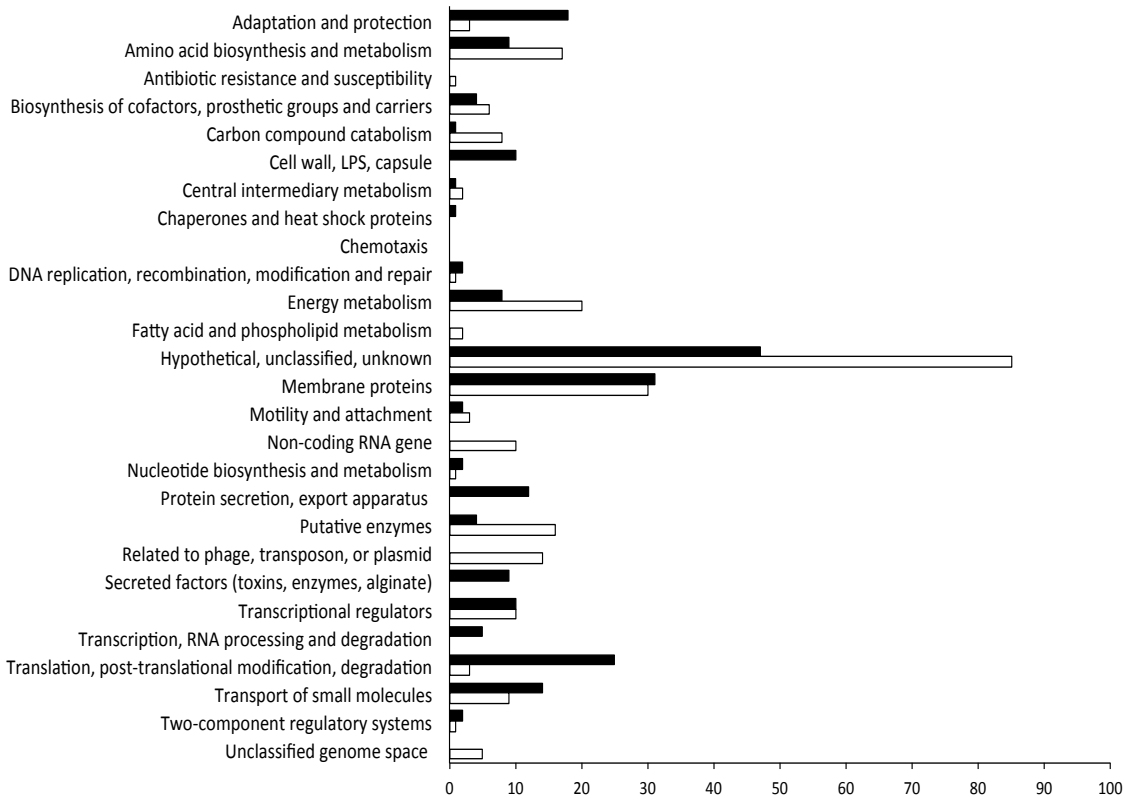


Fig. 9: Functional classification of genes differentially regulated during growth in ASM-Chelex compared to *ex vivo* sputum samples of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4

Differentially regulated genes (pfp < 0.05) are classified according to "Pseudomonas Genome Database" (Winsor *et al.*, 2011). Black bars represent downregulated genes, whereas white bars represent upregulated genes in ASM-Chelex compared to *ex vivo* sputum samples.

Approximately the same amount of membrane protein genes was up- and downregulated with 30 to 31 genes, respectively. The same was observed for transcriptional regulators.

Upregulated were also general stress response regulator sigma factor gene *rpoS* (Suh *et al.*, 1999) and the phosphate uptake regulating transcriptional regulator gene *phoP* und *phoB*. Downregulated were the sigma factor *rpoD* and the choline metabolism regulator *betI*. As previously described the iron dependent regulators *femI*, *pvdS*, *foxI* and *pchR* were repressed. Nearly 40% of the upregulated genes are classified as hypothetical, unclassified, unknown genes. Selected important genes and regulons are discussed in detail in the following sections.

2.2.6 Transcriptional profile of ASM-Chelex vs. ASM

The transcriptome of the growth conditions in ASM-Chelex were compared to ASM. Genes were selected and classified as previously described (Fig. 10).

Only 190 genes were regulated differently during growth in ASM-Chelex compared to ASM. 143 genes were upregulated in ASM-Chelex. Only 47 genes were downregulated. Significant induction was seen for genes classified as membrane protein genes. The same was observed for transcriptional regulator and two-component regulatory system genes. Furthermore, genes related to phage, transposon or plasmid were upregulated. Genes concerning carbon compound catabolism were downregulated as well as putative enzyme genes.

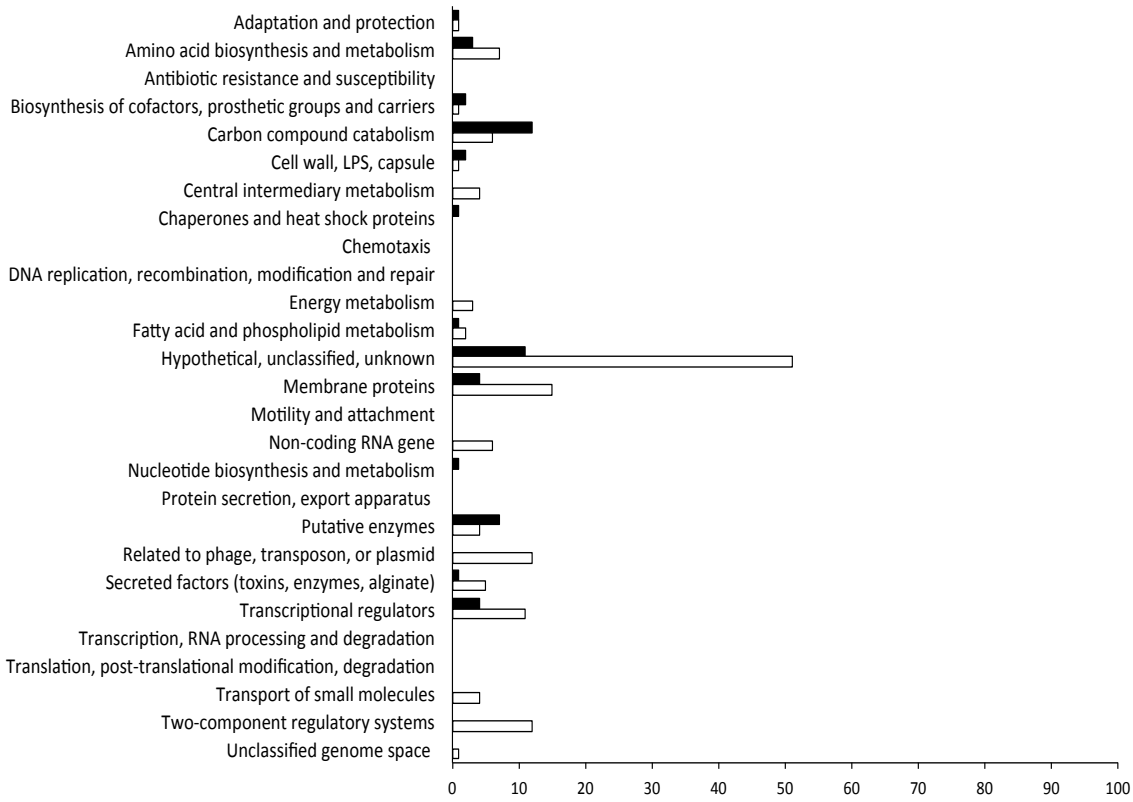


Fig. 10: Functional classification of genes differentially regulated during growth in ASM-Chelex compared to growth in ASM the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4

Differentially regulated genes (pfp < 0.05) are classified according to "Pseudomonas Genome Database" (Winsor *et al.*, 2011). Black bars represent downregulated genes, whereas white bars represent upregulated genes in ASM-Chelex compared to ASM samples.

Furthermore, the transcriptional regulators such as *ptrB* gene an indirect regulator of the type three secretion system (T3SS) and the phosphate uptake regulating transcriptional regulator

gene *phoB* (Wu and Jin, 2005) were upregulated in ASM-Chelex. The iron dependent transcriptional regulators *vreA* and *pchR* and other genes typically induced by iron limitation were upregulated in ASM-Chelex.

2.2.7 Predicted and proven Fur regulon of genes involved in iron acquisition, uptake, storage and metabolism

Since free iron can easily induce the generation of toxic cell damaging radicals its acquisition and storage is tightly regulated. In general, iron acquisition, uptake and storage are directly or indirectly controlled by the global ferric uptake regulator Fur, extracytoplasmic function (ECF) sigma factors or other types of regulators such as two-component regulatory systems and AraC-type regulators in *P. aeruginosa*. Bacteria need to obtain iron in order to grow, to survive, to produce virulence factors and to establish and maintain an infection. Moreover, iron uptake is an energy consuming process. Siderophore production, but also the transport through the outer and cytoplasmatic membrane are both energy driven processes (Braun and Killmann, 1999; Llamas *et al.*, 2008). Therefore, it is even more important to express all genes involved in iron acquisition and uptake only when it is necessary.

With the modified Fig. 11 and Fig. 12 from Cornelis *et al.* (2009) an overview of the genes is given (green and red dashed lines), which were discovered regulated in ASM-Chelex vs. *ex vivo* and their position in the complex Fur regulatory network.

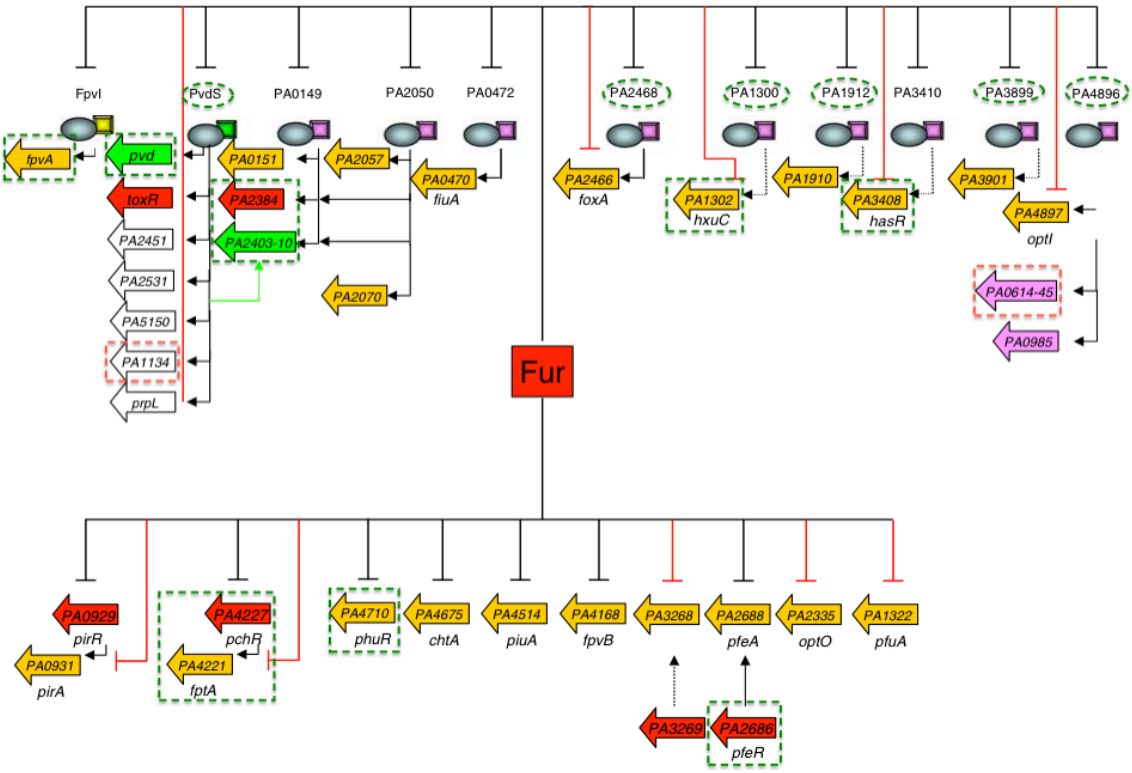


Fig. 11: Direct and indirect control of TonB-dependent genes by Fur modified from Cornelis *et al.* (2009)

The first line shows the ECFs factors that are under control of Fur. The second line shows the additional genes under control of Fur. Furthermore, genes encoding TonB-dependent receptors (yellow), transcription regulators (red) and the genes of the pyoverdine locus (green) are indicated. Arrows indicate activation, while broken lines represent repression. Black lines represent

experimentally verified interactions and red lines are predicted by the van Oeffelen *et al.* (2008) analysis. Stippled lines are suggested, but not experimentally confirmed, interactions. Green dashed lines represent downregulated genes detected in this study, whereas red dashed lines represent upregulated genes in ASM-Chelex vs. *ex vivo*.

Fur acts as repressor of iron uptake genes when Fe^{2+} is bound (Vasil and Ochsner, 1999). The ECF sigma factors frequently consist of three proteins. One is a TonB-dependent outer membrane receptor. The others are a cytoplasmic membrane protein that functions as an antisigma factor and the ECF sigma factor. The outer membrane receptor is involved in transport of the siderophore but moreover it is involved in signal transduction (Llamas *et al.*, 2008; Ogierman and Braun, 2003).

The ECF iron-starvation class sigma factors genes of *pvdS*, *fiuI*, PA1300, *femI*, *foxI*, *fecI* and PA4896 were downregulated in ASM-Chelex vs. *ex vivo* in this study (Fig. 11). But also only predicted Fur regulated genes were also downregulated in ASM-Chelex vs. *ex vivo*, which further confirms the fact that these genes are probably Fur regulated (Fig. 12). This also indicates that the iron limitation is much stronger *in vivo* than in ASM-Chelex.

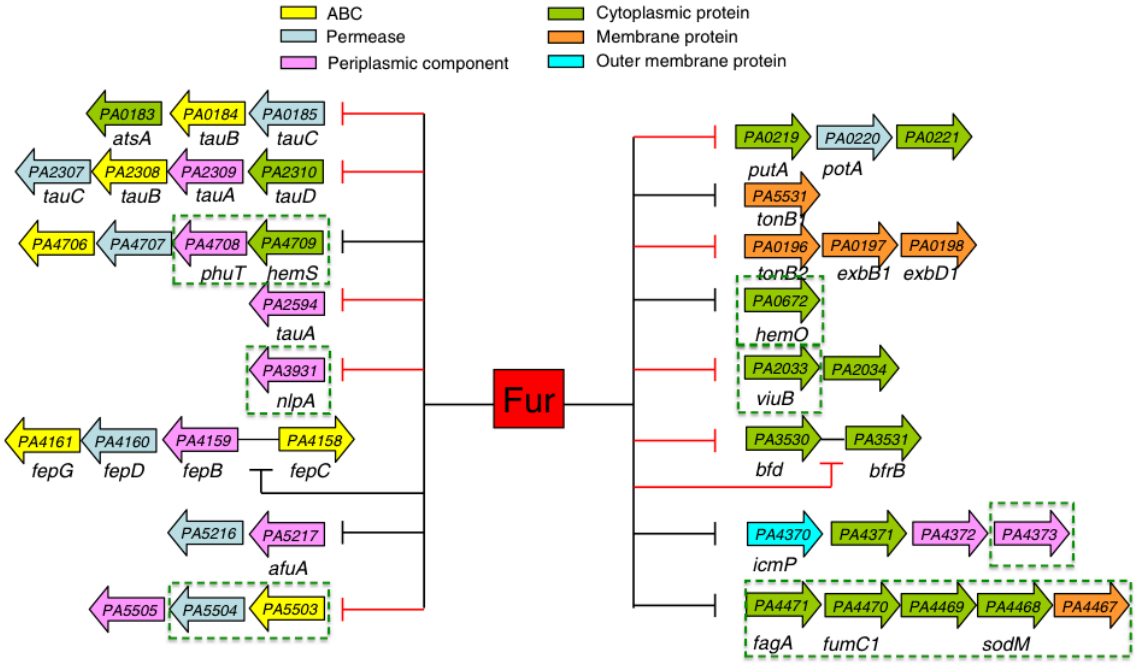


Fig. 12: Predicted Fur regulation of genes involved in iron uptake and storage modified from Cornelis *et al.* (2009)

Non-experimentally proven interactions are shown as red arrows (van Oeffelen *et al.*, 2008). Protein categories are colour coded as indicated in the figure. Green dashed lines represent downregulated genes detected in this study, whereas red dashed lines represent upregulated genes in ASM-Chelex vs. *ex vivo*.

The Fur regulon is a very important regulon under the *ex vivo* CF conditions, since many of the proven and predicted genes of this regulon are upregulated under *ex vivo* conditions.

2.2.8 Iron starvation - a characteristic condition of cystic fibrosis airway *ex vivo* infection and also induced in ASM-Chelex

In order to overcome iron limitation, *P. aeruginosa* produces a wide range of factors such as siderophores, proteases, heme uptake systems and virulence factors such as exotoxin A (Ochsner et al., 2002). Many of these genes were identified in the here presented microarray study comparing the *ex vivo* sputum samples to ASM and ASM-Chelex samples (Cornelis *et al.*, 2009; Klein *et al.*, 2008; Llamas *et al.*, 2008; Ochsner *et al.*, 2002; Palma *et al.*, 2003; Vasil and Ochsner, 1999). A large number of predicted or proven iron regulated genes, in total 101 genes, were differently regulated in our study (Table 3). But neither ASM nor ASM-Chelex was able to induce the same level of iron limitation as the *ex vivo* conditions.

The ECF sigma factor gene encoding for PvdS, which controls the expression of pyoverdine biosynthesis genes and reacts to the presence of the siderophore, is upregulated under *ex vivo* conditions and downregulated in ASM and ASM-Chelex. The majority of iron regulated genes were downregulated in ASM vs. *ex vivo* and ASM-Chelex vs. *ex vivo*. The same expression pattern was observed for the genes encoding for other ECF sigma factors belonging to the iron-starvation class as FiuI, PA1300, FemI, FoxI, FecI and PA4896 were identified, which are involved in ferrichrome uptake, heme uptake, mycobactin/carboxymycobactin uptake, ferrioxamine uptake, iron citrate uptake and siderophore uptake, respectively (Cornelis *et al.*, 2009; Llamas *et al.*, 2008). The pyochelin receptor *fptA* gene and the pyochelin biosynthesis genes, which are regulated by the AraC-type transcriptional regulator PchR, were regulated by the same pattern described before. And also the expression of *tonB1* (PA5531) gene and several genes involved in heme uptake e.g. *hasAp*, *hasR* and PA4708-PA4710 were downregulated in ASM and ASM-Chelex vs. *ex vivo*. Moreover, the genes PA4357-PA4359, which transport ferrous iron, were downregulated.

As expected in ASM-Chelex vs. *ex vivo* the degree of downregulation was less for the majority of genes than in ASM vs. *ex vivo*, which can bee seen for *fptA* which is -41,7-fold (A/EV) and -5,2-fold in (ACh/EV) or *pvdS* with - 71,9-fold (A/EV) and - 37,6-fold in (ACh/EV) (Table 3). Moreover all iron regulated genes were upregulated in ASM-Chelex (ACh/A). This indicates that the degree of iron limitation and the iron starvation rate of bacteria was much higher in ASM-Chelex than in ASM as intended by the design of the ASM-Chelex medium. Iron limitation was definitely induced in ASM-Chelex by Chelex. But the comparison of the transcriptional profile to the *ex vivo* CF conditions reveals also that the iron limitation was much stronger *ex vivo*. This is also supported by the comparison of *ex vivo* vs. LB conditions of selected genes (Bielecki, 2008). When *ex vivo* vs. LB conditions were compared eight of nine genes were upregulated, which were detected under these conditions (Table 3). The highest induction was observed for *hasAP,* which is involved in heme uptake. This indicates that the uptake of heme is a very important factor in iron acquisition under *ex vivo* conditions.

Table 3: Fold change of gene expression of selected* iron regulated genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A) and selected genes of *ex vivo* compared to LB (EV / LB). The qRT-PCR investigated genes are highlighted in bold letters. ECF sigma factors are shown in bold grey letters.

Gene ID[a]*	Gene name[a]	Function[a]	FC A / EV	FC ACh / EV	FC ACh / A	FC EV / LB[c]
Iron starvation:						
PA0472	*fiuI*	probable sigma-70 factor, ECF subfamily	-9,0			
PA0672	*hemO*	heme oxygenase	-133,3	-48,5		
PA0674	*vreA*	VreA	-21,4		8,7	
PA0929		two-component response regulator	-22,6		10,0	
PA0930		two-component sensor	-8,1			
PA1134		Predicted thiol-disulphide oxidoreductase		5,1	6,2	
PA1300		probable sigma-70 factor, ECF subfamily	-42,2	-37,5		
PA1301		probable transmembrane sensor	-14,5	-12,0		
PA1302	*hxuC*	probable heme utilization protein precursor	-5,6	-5,6		
PA1911	*femR*	sigma factor regulator, FemR	-7,4	-5,1		
PA1912	*femI*	ECF sigma factor, FemI	-11,1	-7,7		
PA1922		probable TonB-dependent receptor	-8,8		15,6	
PA2033	*viuB*	catecholate siderophore uptake	-16,4	-12,5		
PA2034		precorrin methylase	-26,8	-24,3		
PA2384		hypothetical Fur-like regulator	-17,8	-15,4		
PA2385	*pvdQ*	3-oxo-C12-homoserine lactone acylase PvdQ	-11,4	-11,4		
PA2386	***pvdA***	**L-ornithine N5-oxygenase**	**-62,5**	**-62,5**		**62,6**
PA2393		probable dipeptidase precursor	-9,2	-9,2		
PA2394	*pvdN*	PvdN	-20,4	-20,7		
PA2395	*pvdO*	PvdO	-6,6	-6,6		
PA2396	***pvdF***	**pyoverdine synthetase F**	**-18,8**	**-24,9**		**10,7**
PA2397	*pvdE*	pyoverdine biosynthesis protein PvdE	-17,8	-17,8		
PA2398	***fpvA***	**ferripyoverdine receptor**	**-101,0**	**-80,0**		**98,7**
PA2399	*pvdD*	pyoverdine synthetase D	-13,1	-14,0		
PA2400	*pvdJ*	PvdJ	-8,6	-8,5		
PA2402		probable non-ribosomal peptide synthetase	-13,3	-13,4		
PA2403		hypothetical protein	-9,2	-5,8		
PA2404		hypothetical protein	-12,1	-10,3		
PA2405		hypothetical protein	-22,1	-13,1		
PA2406		hypothetical protein	-13,5	-13,4		
PA2407		probable adhesion protein	-28,2	-28,5		
PA2408		probable ATP-binding component of ABC transporter	-6,3	-6,8		
PA2409		probable permease of ABC transporter	-10,5	-7,3		
PA2411		probable thioesterase	-20,0	-20,0		
PA2412		conserved hypothetical protein	-29,0	-25,9		
PA2413	*pvdH*	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase	-6,2	-6,0		
PA2424	*pvdL*	PvdL	-7,9	-7,9		
PA2426	***pvdS***	**sigma factor PvdS**	**-71,9**	**-37,6**		**70,4**
PA2427		hypothetical protein			5,2	
PA2467	*foxR*	Anti-sigma factor FoxR	-15,5	-8,6		
PA2468	*foxI*	ECF sigma factor FoxI	-7,9	-4,9		
PA2686	*pfeR*	two-component response regulator PfeR	-5,1	-5,1		
PA3397	*fpr*	ferredoxin--NADP+ reductase	-6,9	-10,0		
PA3407	*hasAp*	heme acquisition protein HasAp	-250,0	-227,3		
PA3408	*hasR*	Haem uptake o.m. receptor HasR precursor	-7,0	-7,0		
PA3530	*bfd*	bacterioferritin-associated ferredoxin			10,2	
PA3531	*bfrB*	bacterioferritin	8,4		-4,3	
PA3768		probable metallo-oxidoreductase	-5,4			
PA3899	*fecI*	probable sigma-70 factor, ECF subfamily	-20,7	-16,7		
PA4221	***fptA***	**Fe(III)-pyochelin o.m. receptor precursor**	**-41,7**	**-5,2**	**8,0**	
PA4222		probable ATP-binding component of ABC transporter	-4,8			
PA4223		probable ATP-binding component of ABC transporter	-23,4		5,8	
PA4224	***pchG***	**pyochelin biosynthetic protein PchG**	**-76,3**		**18,0**	
PA4225	*pchF*	pyochelin synthetase	-19,2	-4,4	4,3	
PA4226	*pchE*	dihydroaeruginoic acid synthetase	-47,8		11,9	
PA4227	*pchR*	transcriptional regulator PchR		-13,9	5,1	16,8
PA4228	*pchD*	pyochelin biosynthesis protein PchD	-27,9		6,5	

Continued:

Gene ID[a]	Gene name[a]	Function[a]	FC A / EV	FC ACh / EV	FC ACh / A	FC EV / LB[c]
PA4229	*pchC*	pyochelin biosynthetic protein PchC	-45,7	-7,7	5,9	
PA4230	*pchB*	salicylate biosynthesis protein PchB	-38,8		9,9	
PA4231	*pchA*	salicylate biosynthesis isochorismate synthase	-18,4	-11,3		
PA4357		conserved hypothetical protein	-23,4	-5,1		
PA4358		probable ferrous iron transport protein	-63,3	-5,4	4,6	
PA4859		conserved hypothetical protein	-9,6		11,6	
PA4370	*icmP*	Insulin-cleaving metalloproteinase o.m. protein precursor	-6,1			
PA4373		hypothetical protein	-8,2	-4,9		
PA4467		hypothetical protein	-38,2	-31,4		
PA4468	*sodM*	superoxide dismutase	-20,2	-15,6		11,9
PA4469		hypothetical protein	-14,1	-10,7		
PA4470	*fumC1*	fumarate hydratase	-42,4	-24,7		34,6
PA4471		hypothetical protein	-6,1	-6,1		
PA4515	*piuC*	Uncharacterized iron-regulated protein	-6,8	-5,4		
PA4570		HUU; weak similarity to IclR repressor family	-43,3	-5,1	8,4	
PA4708	*phuT*	Heme-transport protein, PhuT	-7,2	-8,1		
PA4709		probable hemin degrading factor	-7,4	-15,7		
PA4710	*phuR*	Haem/Haemoglobin uptake o.m. receptor PhuR precursor	-9,7	-25,8		
PA4834		hypothetical protein	-15,0			
PA4835		hypothetical protein	-36,9	-9,2		
PA4836		hypothetical protein	-27,9	-5,2	5,4	
PA4837		probable o.m. protein precursor	-43,7	-6,1	7,2	
PA4895		probable transmembrane sensor	-6,0	-6,0		
PA4896		probable sigma-70 factor, ECF subfamily	-21,5	-19,0		19,1
PA5217		probable binding protein component of ABC iron transp.	-5,8		5,1	
PA5531	*tonB1*	TonB protein	-5,5		6,2	
Regulated by PA4896[b] (all genes classified as "related to phage, transposon, or plasmid"):						
PA0614		Putative holin		14,4	6,8	
PA0622		R-type pyocin, related to P2 phage; tail sheath		6,2		
PA0623		R-type pyocin, related to P2 phage; tail tube		7,6	8,2	
PA0624		R-type pyocin, related to P2 phage		10,6	7,3	
PA0627		R-type pyocin, related to P2 phage; tail formation		7,1	7,6	
PA0631		Related to phage, lysis control		5,7	5,7	
PA0633		F-type pyocin, related to λ phage; major tail protein		5,5	5,2	
PA0634		F-type pyocin, related to λ phage		11,4	8,7	
PA0635		F-type pyocin, related to λ phage		29,2	22,6	
PA0636		F-type pyocin, related to λ phage; tail length determination		6,4	5,3	
PA0637		F-type pyocin, related to λ phage; tail formation		6,2	5,4	
PA0638		F-type pyocin, related to λ phage; tail formation conserved		6,2		
PA0639		F-type pyocin, related to λ phage; tail formation		6,0	5,6	
PA0647		hypothetical protein		7,2	4,0	
PA0648		hypothetical protein		18,2	19,3	
Predicted Fur regulation by (van Oeffelen *et al.*, 2008):						
PA5503		probable ATP-binding component of ABC transporter		-5,0		
PA5504		D-methionine ABC transporter membrane protein		-5,8		
PA3931	*nlpA*	conserved hypothetical protein		-6,3		
PA4296	*pprB*	two-component response regulator	15,7		-4,6	-34,3

[a] Gene ID, gene name, function and PseudoCAP Function Class (PCFC) are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

[b] Functions for genes regulated by PA4896 are annotated according to (Llamas *et al.*, 2008)

[c] The study was performed together with Piotr Bielecki (HZI). Selected genes from the comparison EV/ LB are shown (Bielecki, 2008).

* Several studies and the ProdoNet data base were applied to obtain the list of iron regulated genes shown here (Cornelis *et al.*, 2009; Klein *et al.*, 2008; Llamas *et al.*, 2008; Ochsner *et al.*, 2002; Palma *et al.*, 2003; Vasil and Ochsner, 1999).

A total of 78 genes were regulated in ASM-Chelex vs. *ex vivo*. 58 genes were downregulated and 16 genes were induced. These 16 genes are mostly characterised as hypothetical genes and especially upregulated in ASM-Chelex. The majority of upregulated genes (PA0614-PA0648) were regulated by PA4896 (van Oeffelen *et al.*, 2008). PA4896 is 63 % similar to *E. coli* FecI. Llamas *et al.* 2008 proposed that PA4896 is involved in siderophore uptake. They

confirmed that the PA4896 regulated genes encoded for the pyocins P2 and F2, which we also identified as upregulated under ASM-Chelex conditions.

It cannot be excluded that the pyocin induction was due to a general stress response. But on the other hand pyocin S2 (PA1150), which is normally induced by SOS-response (Llamas *et al.*, 2008; Michel-Briand and Baysse, 2002), is not induced under the investigated conditions.

Llamas *et al*. proposed that by using pyocins *P. aeruginosa* is killing competing bacteria, while using the system for "stealing" their siderophores. Why this system was upregulated only in ASM-Chelex has to be further investigated.

The genes PA4834-PA4837 were downregulated in ASM and ASM–Chelex, which means that they are upregulated under *ex vivo* conditions. These genes are predicted to encode a novel siderophore system (Bielecki, 2008). PA4837 encodes for a predicted TonB dependent siderophore receptor (Winsor *et al.*, 2011). Moreover, PA4837 has been shown to be upregulated in *P. aeruginosa* in *in vivo* burn wound patients' samples, in 10% CF sputum medium and in an *in vivo* peritoneal rat infection model (Bielecki *et al.*, 2011; Mashburn *et al.*, 2005; Palmer *et al.*, 2005).

2.2.9 Other genes closely connected to iron metabolism

Unexpectedly the PQS biosynthesis genes were downregulated under *ex vivo* conditions and upregulated in ASM and ASM-Chelex, especially the genes *pqsA* and *pqsD* (Table 4). The *phnAB* operon (Fig. 13) encoding for anthranilate biosynthesis was also upregulated in ASM-Chelex vs. *ex vivo* and ASM-Chelex vs. ASM. Several studies suggest that PQS is one of the molecules closely connected to iron metabolism (Bredenbruch *et al.*, 2005; Diggle *et al.*, 2007b; Schertzer *et al.*, 2009). The PQS quorum sensing system uses 2-heptyl-3-hydroxyl-4-quinolone as signalling molecule (Diggle *et al.*, 2007b). As well as being a signalling molecule PQS is also predicted to have iron chelating ability and thereby leading to activation of iron acquisition genes and genes necessary for oxidative-stress protection (Table 3). But the induction in ASM and ASM-Chelex probably correlates with the previously reported fact that PQS synthesis might be triggered by the high availability of aromatic amino acids (Haussler and Becker, 2008; Palmer *et al.*, 2007a; Palmer *et al.*, 2005). This might indicate that the availibility of aromatic amino acids might be higher in ASM and ASM-Chelex than under *ex vivo* conditions.

In vivo PQS biosynthesis might be also decreased due to high concentrations of PQS in the lung (Collier *et al.*, 2002), compared to ASM or ASM-Chelex. Clearly, the iron level in ASM-Chelex is reduced to ASM but clearly not as low as in the *in vivo* environment.

The predicted *pcaIJF*, *pcaTBDC* and *antRABC* operon genes (Fig. 13) are also involved in anthranilate metabolism and degradation and were upregulated in ASM vs. *ex vivo*, but downregulated in ASM-Chelex vs. ASM.

The *antRABC* operon is predicted to be regulated by small regulatory RNAs *prrF1* and *prrF2*. PrrF1 and PrrF2 RNAs contribute to iron homeostasis under depleted conditions by inducing degradation of mRNAs encoding for iron-containing proteins (Cornelis *et al.*, 2009; Oglesby *et al.*, 2008; Vasil, 2007). Oglesby *et al.* (2007) demonstrated that anthranilate availability is tightly regulated for either PQS or energy production depending on the iron level of the cell. During iron limitation *antABC* is repressed by PrrF, which can be observed for ASM-Chelex (ACh/A).

Table 4: Fold change of gene expression of selected PQS biosynthesis, anthranilate metabolism and sigma factor genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 – 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) CF patients' samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A).

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
PQS-Biosynthesis:					
PA0996	*pqsA*	probable coenzyme A ligase	5,3	34,3	6,4
PA0997	*pqsB*	Homologous to beta-keto-acyl-acyl-carrier protein synthase		30,6	14,7
PA0998	*pqsC*	Homologous to beta-keto-acyl-acyl-carrier protein synthase		39,0	13,1
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	4,1	34,7	8,4
PA1000	*pqsE*	Quinolone signal response protein		23,5	12,9
PA1001	*phnA*	anthranilate synthase component I		34,1	19,5
PA1002	*phnB*	anthranilate synthase component II		26,7	10,9
Anthranilate-Metabolism:					
PA0226	*pcaI*	probable CoA transferase, subunit A	95,1		-64,1
PA0227	*pcaJ*	probable CoA transferase, subunit B	53,3		-42,6
PA0228	*pcaF*	beta-ketoadipyl CoA thiolase PcaF	55,6		-54,9
PA0229	*pcaT*	dicarboxylic acid transporter PcaT	5,6		-5,4
PA0230	*pcaB*	3-carboxy-cis,cis-muconate cycloisomerase	6,6		-6,5
PA0231	*pcaD*	beta-ketoadipate enol-lactone hydrolase	10,6		-10,2
PA0232	*pcaC*	gamma-carboxymuconolactone decarboxylase	31,11		-19,6
PA2507	*catA*	catechol 1,2-dioxygenase	82,0		-103,1
PA2508	*catC*	muconolactone delta-isomerase	159,8		-56,8
PA2509	*catB*	muconate cycloisomerase I	55,1		-51,5
PA2511	*antR*	probable transcriptional regulator	21,8		-14,1
PA2512	*antA*	anthranilate dioxygenase large subunit	102,5		-80,0
PA2513	*antB*	anthranilate dioxygenase small subunit	241,7		-65,8
PA2514	*antC*	anthranilate dioxygenase reductase	83,5		-80,0
Sigma factor:					
PA3622	*rpoS*	sigma factor RpoS	4,6	9,8	
PA0576	*rpoD*	sigma factor RpoD		-4,9	

In ASM vs. *ex vivo antABC* is induced. This indicates that iron repleted conditions under which both PQS synthesis and anthranilate degradation for energy production take place seem to be present in ASM (Table 4) from the microarray results, which needs to be further verified by other techniques.

Anthranilate metabolism is further regulated by the AHL dependent LasI / LasR and RhlI / RhlR quorum sensing systems and in addition by AntR the anthranilate-dependent activator (Chugani and Greenberg, 2010; Oglesby *et al.*, 2008; Williams and Camara, 2009).

The alternative sigma factor RpoS, which was induced in ASM vs. *ex vivo* and ASM-Chelex vs. *ex vivo*, regulates also AHL quorum sensing dependent genes such as virulence genes as pyocyanin and exotoxin A, during the onset of stationary phase (Potvin *et al.*, 2008; Schuster *et al.*, 2004). In contrast *rpoD*, which gene product mainly regulates the expression of housekeeping genes, was downregulated in ASM-Chelex vs. *ex vivo*.

PQS and PQS biosynthesis and metabolism and degradation of anthranilate are potent indicators of the iron status of the cell and thereby for the surrounding environment.

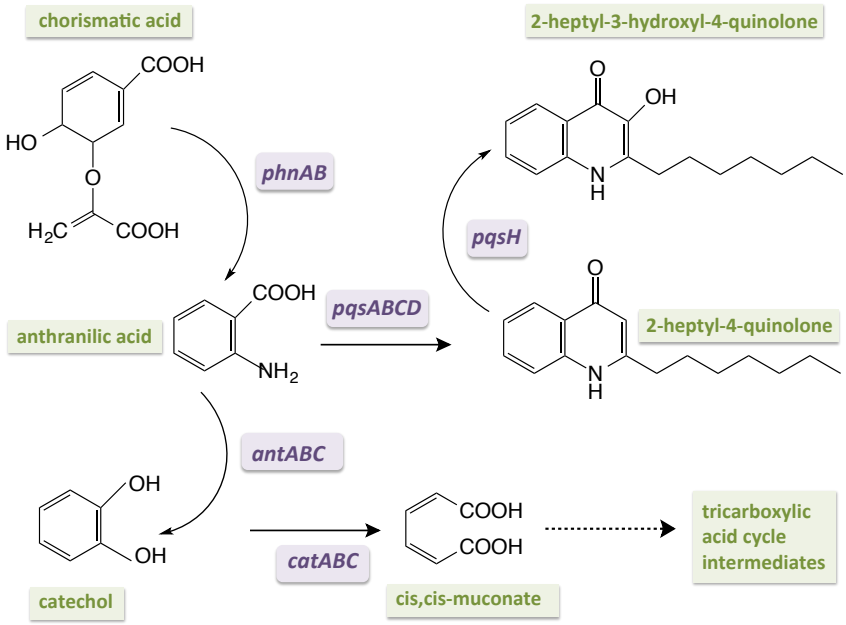


Fig. 13: Schematic overview of PQS synthesis and anthranilate degradation (modified after (Bundy *et al.*, 1998; Farrow and Pesci, 2007)

Chorismatic acid is converted by the anthranilate synthase (encoded by *phnAB)* to anthranilic acid. Anthranilic acid can be converted by gene products encoded by the *pqsABCD* operon to 2-heptyl-4-quinolone. The reaction to 2-heptyl-3-hydroxyl-4-quinolone (PQS) is catalysed by the gene product of *pqsH*.

Moreover, anthranilic acid can be degraded by the anthranilate dioxygenase (encoded by *antABC*) to catechol, which can be further converted by catechol dioxygenase encoded by *catABC* operon to cis,cis-muconate. Muconate can be further degraded to tricarboxylic acid cycle intermediates via β-ketoadipate pathway.

2.2.10 Sulphur, molybdenum, zinc and phosphate are important factors for survival in cystic fibrosis lung

Microelements such as sulphur, molybdenum and zinc are important cofactors of enzymes necessary for infection and survival. But in ASM and ASM-Chelex the upregulation of genes encoding proteins involved in the uptake of none of these factors was observed (Table 5). Therefore, these uptake systems and binding proteins were upregulated only under *ex vivo* conditions. A recent study suggests that zinc is also taken up via pyochelin, but the affinity for iron is significantly higher (Brandel *et al.*, 2012). Enzymes important under infection conditions, such as LasA protease and LasB elastase are zinc metallo proteases and need zinc to be functional (Olson and Ohman, 1992). But zinc limitation was only observed under *ex vivo* conditions not in ASM nor ASM-Chelex.

The sulphur supply during CF infection might be replenished by desulphuration of mucin residues, which are highly sulphated and sialylated (Robinson *et al.*, 2012; Tralau *et al.*, 2007). Besides the sulphur pool might be refilled by uptake of the sulphur containing amino acids methionine and cysteine. In ASM and ASM-Chelex sulphur is probably obtained by the uptake

of mucine, methionine or cysteine. No limitation of sulphur was observed after 24 h of growth in ASM and ASM-Chelex.

Furthermore, a recent study showed that CysB the central regulator of sulphur metabolism in *P. aeruginosa* is crucial for PvdS expression (Imperi *et al.*, 2010). One well-known connection between iron and sulphur metabolism are the iron-sulphur clusters, which are essential prosthetic groups for electron transfer, gene regulation and environmental sensing. Therefore, assembly is carefully regulated to avoid toxicity of free iron and sulphide (Ayala-Castro *et al.*, 2008). On the other hand energy is saved, when uptake and assembly are coregulated. This might be the connection between CysB and PvdS and hence between iron and sulphur metabolism.

Table 5: Fold change of gene expression of selected sulphur, molybedenum, zinc and phosphate regulated genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 – 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A).

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
Sulphur:					
PA0283	*sbp*	sulfate-binding protein precursor		-6,0	
Molybdenum:					
PA3441		probable molybdopterin-binding protein	-5,1		
PA3915	*moaB1*	molybdopterin biosynthetic protein B1		-15,4	
Zinc:					
PA5500	*znuC*	zinc transport protein ZnuC	-6,5		
PA5503		probable ATP-binding component of ABC transporter		-5,0	
Phosphate:					
PA1178	oprH	PhoP/Q, low Mg2+ inducible o.m. prot. H1 prc.		5,6	
PA1179	phoP	two-component response regulator PhoP		7,6	
PA1180	phoQ	two-component sensor PhoQ		9,4	
PA2657		probable two-component response regulator			4,8
PA3280	*oprO*	Pyrophosphate-specific outer membrane porin OprO precursor	-5,5		21,7
PA3382	*phnE*	phosphate transport protein PhnE		9,3	9,6
PA5360	*phoB*	two-component response regulator PhoB		38,4	41,4
PA5365	*phoU*	phosphate uptake regulatory protein PhoU		7,3	14,2
PA5366	*pstB*	ATP-binding component of ABC phosphate transporter		15,4	33,5
PA5367	*pstA*	membrane protein component of ABC phosphate transporter		12,6	16,7
PA5368	*pstC*	membrane protein component of ABC phosphate transporter		7,4	7,4
PA5369	*pstS*	phosphate ABC transporter, periplasmic phosphate-binding protein		16,5	22,3

Molybdenum is needed for the molybdenum cofactor biosynthesis. The cofactor is an important part of enzymes of the denitrification such as NarG and NapA (Zumft, 1997). Both enzymes are part of the dissimilatory nitrate reductases. *P. aeruginosa* harbours two systems one is the NarGHI and the second is NapABC. The *napA* gene and the *napABC* operon were detected upregulated in ASM vs. *ex vivo* and ASM-Chelex vs. *ex vivo* (Table 6). Molybdenum is an important component for the bacterial energy production as molybdenum cofactor, but molybdenum limitation was only observed under *ex vivo* conditions.

In ASM-Chelex (ACh/EV; ACh/A) the phosphate uptake system genes such as the two-component response regulator gene *phoB*, the phosphate uptake regulatory protein gene *phoU*, the predicted operon *pstBAC* genes and the periplasmic phosphate-binding protein gene *pstS*

were induced (Hancock *et al.*, 1992; Siehnel *et al.*, 1992) (Table 5). Besides the predicted *oprH*, *phoPQ* operon, which is associated with phosphate metabolism (Groisman *et al.*, 1989), was upregulated under the ASM-Chelex vs. *ex vivo* conditions. Phosphate starvation is reported to induce the virulence factor production of *P. aeruginosa* associated with phosphate, quorum sensing, and iron signalling in a *Caenorhabditis elegans* model system (Zaborin *et al.*, 2009).

Zaborin *et al.* (2009) also observed a connection between phosphate depletion and increased pyoverdine production, which led to a more lethal phenotype in *C. elegans*. The response to phosphate depletion could therefore be investigated in ASM-Chelex.

Only the gene *oprO*, which is only expressed under phosphate-starvation conditions, was induced under ex *vivo* conditions (A/EV) (Hancock *et al.*, 1992; Siehnel *et al.*, 1992). In this study phosphate depletion in ASM-Chelex (ACh/EV; ACh/A) might be an effect indirectly connected to the Chelex resin. Previous studies describe the interaction of the divalent phosphate anion HPO_4^{2-} with polyvalent metal ions such as Cu (II), Fe(III), Mo(IV) (Wu *et al.*, 2007; Zhao and Sengupta, 1998). Moreover, Wu *et al.* (2007) describe the efficient removal of phosphate by lanthanum (III) bound to Chelex resin from water. One can discuss if the metal ions in ASM-Chelex, which are chelated by the Chelex resin, were acting in combination with Chelex probably as very efficient phosphate scavengers. Thereby probably introducing the observed phosphate limitation in ASM-Chelex.

2.2.11 Energy metabolism *ex vivo*, in ASM and in ASM-Chelex

P. aeruginosa is capable to use a wide range of carbon and energy sources for the production of energy. Energy conservation as adenosine triphosphate (ATP) is possible via aerobic or anaerobic respiration or fermentation of arginine or pyruvate (Davies *et al.*, 1989; Eschbach *et al.*, 2004; Vander Wauven *et al.*, 1984; Yoon *et al.*, 2002; Zannoni, 1989)

For the complete oxidation of organic compounds during respiration an external electron acceptor is necessary. Electrons are transferred to the external electron acceptor via an electron transport chain, which translocates protons into the periplasm and thereby generates an electrochemical gradient (proton motive force, PMF) across the cytoplasmic membrane, which is essential for ATP production. Without an available electron acceptor, fermentation of organic substances via substrate-level phosphorylation can be used for ATP synthesis.

During growth under *ex. vivo* conditions (A/EV ; ACh/EV) parts of the *narK1K2GHJI* operon, which encodes for NarGHI and the two transmembrane proteins $NarK_1K_2$, were upregulated (Table 6 and Fig. 14). This clearly indicates that *P. aeruginosa* uses the *narK1K2GHJI* operon to obtain energy facing the microaerobic to anaerobic conditions *in vivo.*

Only $NarK_2$ seems to be essential for nitrate/nitrite transport (Sharma *et al.*, 2006). NarJ is involved in assembly of the enzyme, but is not part of the nitrate reductase (Zumft, 1997). Expression of the *narK1K2GHJI* operon depends on the oxygen-sensing regulator Anr, the NO-sensing regulator Dnr and the nitrate response two-component system NarX-NarL, respectively (Schreiber *et al.*, 2007). The observed regulation is in accordance with literature, which described microaerobic till anaerobic growth conditions in the CF airways (Moreau-Marquis *et al.*, 2008; Worlitzsch *et al.*, 2002).

Neither ASM nor ASM-Chelex were incubated under anaerobic conditions but under aerobic conditions with limited oxygenation. Both media were supplemented with nitrate and nitrite concentrations of 260 µM and 130 µM, respectively.

In accordance to aerobic incubation and nitrate/nitrite availability the second dissimilatory nitrate reductase operon *napABC* was induced in ASM and ASM-Chelex (A/EV ; ACh/EV). The periplasmatic NapABC is not involved in translocation of protons and therefore not used for energy generation as NarGHI. In contrast NapABC reduces nitrate under both aerobic and anaerobic conditions (Davies *et al.*, 1989). Furthermore, it might function as terminal oxidase maintaining the redox balance of the cell during transitions from aerobic to anaerobic conditions or aerobic denitrification (Philippot and Hojberg, 1999).

The conditions *in vivo* might supplement more nitrate. Physiological nitrate and nitrite concentrations in patient's sputum vary from 73 µM to 792 µM (Grasemann *et al.*, 1998; Hassett, 1996; Linnane *et al.*, 1998; Palmer *et al.*, 2007b).

Table 6: Fold change of gene expression of selected energy metabolism genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 – 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A).

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
Denitrification:					
PA0105	*coxB*	cytochrome c oxidase, subunit II	23,3	26,2	
PA0106	*coxA*	cytochrome c oxidase, subunit I	35,7	42,8	
PA0107		conserved hypothetical protein	25,0	33,2	
PA0108	*coIII*	cytochrome c oxidase, subunit III	43,7	48,5	
PA0524	*norB*	nitric-oxide reductase subunit B	-6,9	-6,9	
PA1172	*napC*	cytochrome c-type protein NapC	5,6		
PA1173	*napB*	cytochrome c-type protein NapB precursor	6,9	5,0	
PA1174	*napA*	periplasmic nitrate reductase protein NapA	28,3	16,0	
PA1175	*napD*	NapD protein of periplasmic nitrate reductase	8,0	4,9	
PA1176	*napF*	ferredoxin protein NapF	15,0	12,3	
PA1177	*napE*	periplasmic nitrate reductase protein NapE	26,8	35,0	
PA1555		probable cytochrome c (cbb3-type)		12,4	5,9
PA1556		probable cytochrome c oxidase subunit (cbb3-type)		8,5	5,9
PA1557		probable cytochrome oxidase subunit (cbb3-type)	4,2	10,6	
PA3873	*narJ*	respiratory nitrate reductase delta chain	-8,0	-8,4	
PA3874	*narH*	respiratory nitrate reductase beta chain	-7,8	-7,8	
PA3876	*narK2*	nitrite extrusion protein 2	-12,6	-11,7	
PA3877	*narK1*	nitrite extrusion protein 1	-13,3	-14,0	
PA4587	*ccpR*	cytochrome c551 peroxidase precursor	7,1	6,3	
PA4810	*fdnI*	nitrate-inducible formate dehydrogenase, gamma subunit	12,9	10,0	
PA4811	*fdnH*	nitrate-inducible formate dehydrogenase, beta subunit	20,4	17,1	
PA4812	*fdnG*	formate dehydrogenase-O, major subunit	28,6	19,7	
Arginine fermentation:					
PA5173	*arcC*	carbamate kinase	-4,6		
Pyruvate metabolism:					
PA2382	*lldA*	L-lactate dehydrogenase		-5,0	
PA3415		probable dihydrolipoamide acetyltransferase	12,0	16,7	
PA3416		probable pyruvate dehydrogenase E1 component, beta chain	8,2	9,3	
PA3417		probable pyruvate dehydrogenase E1 component, alpha subunit	5,8	9,4	
Central intermediary metabolism:					
PA5288	*glnK*	nitrogen regulatory protein P-II 2	4,6		

For the conversion from nitrite to nitric oxide by the nitrite reductase NirS, a cytochrome cd_1 reductase located in the periplasm, no significant change in expression (pfp > 0.05) was

observed. The next step the reduction of highly toxic nitric oxide to nitrous oxide is performed by the membrane bound nitric oxide reductase NorCB. The gene *norB*, encoding for a cytochrome b (Arai *et al.*, 1995), was upregulated under *ex vivo* conditions (A/EV ; ACh/EV). Nitrous oxide is converted to molecular nitrogen by the nitrous oxide reductase NosZ, which is located in the periplasm. The expression of *nosZ* gene was not significantly changed (pfp > 0.05) under the investigated conditions.

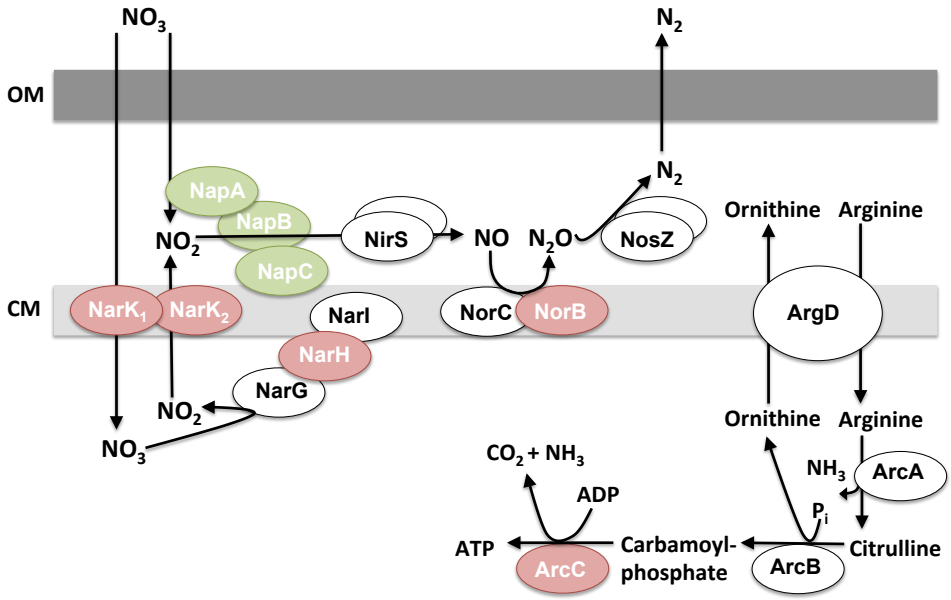


Fig. 14: Schematic depiction of denitrification and arginine fermentation pathways in *P. aeruginosa*

Nitrate is transported via $NarK_2$ into the bacterial cell, where NarGHI catalyses the first step in denitrification, the reduction of nitrate to nitrite. Nitrate can be reduced to nitrite in the periplasm by NapABC. The periplasmic NirS reductase reduces nitrite to nitric oxide, followed by the conversion to nitrous oxide via the NorCB reductase. As final step the NosZ reductase converts nitrous oxide to molecular nitrogen.

The transport of arginine into the cytoplasm via ArcD is coupled with the export of ornithine. The conversion of arginine via the arginine deaminase pathway is catalysed by ArcA, ArcB and ArcC and provides one molecule of ATP per one molecule arginine (Gamper *et al.*, 1991).

Cytoplasmic membrane (CM) and outer membrane (OM) are indicated. The up- (red), downregulation (green) and stable (white) expression of the genes encoding for the depicted proteins is shown for the *ex vivo* sputum sample compared to ASM and ASM-Chelex.

The predicted nitrate-inducible formate dehydrogenase operon genes *fdnIHG* was induced in ASM and ASM-Chelex (A/EV ; ACh/EV). In accordance with previous studies the cytochrome c oxidase operon *coxBA*, PA0107 and *coIII* was upregulated in ASM and ASM-Chelex (A/EV ; ACh/EV). The *cox* operon coding for the aa_3-type low-affinity oxidase is shown to be preferentially expressed in the stationary phase in an aerobic environment and is moreover RpoS regulated (Alvarez-Ortega and Harwood, 2007; Schuster *et al.*, 2004). Moreover, the cbb_3-type 2 oxidase operon was upregulated in ASM-Chelex (ACh/EV ; ACh/A). The cbb_3-type 2 oxidase is predicted to have a high affinity for oxygen and is expressed at low oxygen levels (Alvarez-Ortega and Harwood, 2007; Kawakami *et al.*, 2010). In addition, the cbb_3-type 2

oxidase operon is positively regulated by Anr and RoxSR. This is indicating that oxygen-limiting conditions were also present in the ASM-Chelex culture.

Only the gene *arcC* was detected upregulated under *ex vivo* conditions (A/EV) of the genes essential for arginine fermentation of the *arcDABC* operon (Fig. 14). The carbamate kinase ArcC, which catalyses the reaction of carbamoylphosphate to ammonia and carbon dioxide and leads to the generation of ATP (Vander Wauven *et al.*, 1984), was downregulated in ASM and therefore upregulated under *ex vivo* conditions. Expression of the operon depends on oxygen limitation and Anr (Galimand *et al.*, 1991; Gamper *et al.*, 1991). Besides, transcription of the operon is stimulated by the presence of exogenous arginine via the arginine responsive regulator ArgR (Lu *et al.*, 1999).

The genes involved in pyruvate metabolism as the predicted pyruvate dehydrogenase operon PA3415-PA3417 were upregulated in ASM and ASM-Chelex. In contrast the L-lactate dehydrogenase *lldA* was upregulated *ex vivo* (ACh/EV).

The comparison of ASM and ASM-Chelex with the *ex vivo* conditions, especially the expression of the *narGHI* operon, the *arcC* gene and the *lldA* gene indicate that energy generation via denitrification, arginine fermentation and pyruvate fermentation are a very important source of energy generation *in vivo*.

2.2.12 Nutrient sources in the *ex vivo* sputum sample – metabolism, biosynthesis and uptake

Closely connected to energy generation are metabolism and uptake. The question remains, which nutrient sources allow *P. aeruginosa* to reach concentrations of ≥ 10^8 bacteria/ml sputum (Palmer *et al.*, 2007a; Son *et al.*, 2007). Upregulated under *ex vivo* conditions (ACh/EV) was the amino acid transport gene *aroP1*, which is especially involved in the transport of aromatic amino acids. In contrast, five amino acid transport genes including the *aroP2* gene were upregulated in ASM and ASM-Chelex (Table 7). Furthermore, genes encoding for the amino acid biosynthesis and metabolism proteins were induced in ASM and ASM-Chelex. This is indicating that amino acids are predominantly used in ASM and ASM-Chelex whereas during *ex vivo* conditions other nutrients are preferred.

The *iscS* gene an L-cysteine desulphurase, which degrades cysteine to pyruvate, was upregulated *ex vivo* (ACh/EV). The genes of the methionine and SAM metabolism were upregulated under *ex vivo* conditions (A/EV ; ACh/EV). This indicates the importance of methionine and the formation of SAM under *in vivo* conditions. The gene *glyA3* of the glycin cleavage system, which is necessary for the formation of N^5, N^{10}-methylene-tetrahydrofolate out of serine and glycine, was upregulated under *ex vivo* conditions (A/EV ; ACh/EV).

The strongest induction of 417-fold was seen for the tRNA gene of serine followed by 328-fold induction of tRNA gene of cysteine in ASM-Chelex vs. *ex vivo*. That indicates that *P. aeruginosa* is probably more active in proteins translation in ASM and ASM-Chelex than under *ex vivo* conditions.

Table 7: Fold change of gene expression of gene expression of selected metabolism genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 – 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A).

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
Amino acid transport:					
PA0866	*aroP2*	aromatic amino acid transport protein AroP2	6,4	21,6	
PA1339		amino acid ABC transporter ATP binding protein	6,7	4,7	
PA1340		amino acid ABC transporter membrane protein	10,6	9,4	
PA1342		probable binding protein component of ABC transporter	12,1	9,5	
PA3000	*aroP1*	aromatic amino acid transport protein AroP1		-5,8	
PA5153		amino acid (lysine/arginine/ornithine/histidine/octopine) ABC transporter periplasmic binding protein	3,8	6,3	
Amino acid biosynthesis and metabolism:					
PA0132		beta-alanine--pyruvate transaminase	5,4	5,0	
PA0782	*putA*	proline dehydrogenase PutA	-6,1		
PA0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase	13,7	29,3	
PA0870	*phhC*	aromatic amino acid aminotransferase		5,8	
PA0871	*phhB*	pterin-4-alpha-carbinolamine dehydratase	4,5	12,8	
PA0872	*phhA*	phenylalanine-4-hydroxylase		9,8	
PA1337	*ansB*	glutaminase-asparaginase	8,9	7,0	
PA1338	*ggt*	gamma-glutamyltranspeptidase precursor	5,5		
PA1999	*dhcA*	DhcA, dehydrocarnitine CoA transferase, subunit A		8,8	6,4
PA2000	*dhcB*	DhcB, dehydrocarnitine CoA transferase, subunit B		7,3	4,8
PA2247	*bkdA1*	2-oxoisovalerate dehydrogenase (alpha subunit)		12,7	
PA2248	*bkdA2*	2-oxoisovalerate dehydrogenase (beta subunit)		8,2	
PA2249	*bkdB*	branched-chain alpha-keto acid dehydrogenase		10,0	
PA3118	*leuB*	3-isopropylmalate dehydrogenase	4,9		
PA3120	*leuD*	3-isopropylmalate dehydratase small subunit	13,3		-9,7
PA3121	*leuC*	3-isopropylmalate dehydratase large subunit	12,1		-10,5
PA3216		probable aspartate-semialdehyde dehydrogenase	10,4	13,3	
PA3418	*ldh*	leucine dehydrogenase	10,4	16,4	
PA3570	*mmsA*	methylmalonate-semialdehyde dehydrogenase	3,9	4,7	
PA3792	*leuA*	2-isopropylmalate synthase	29,7		-20,1
PA3814	*iscS*	L-cysteine desulphurase (pyridoxal phosphate-dependent)		-5,8	
PA5410	*gbcA*	GbcA	8,3		
PA5429	*aspA*	aspartate ammonia-lyase		4,9	13,3
Methionine and SAM metabolism:					
PA0316	*serA*	D-3-phosphoglycerate dehydrogenase	-6,3	-15,2	
PA0430	*metF*	5,10-methylenetetrahydrofolate reductase		-5,5	
PA0546	*metK*	methionine adenosyltransferase	-8,4	-8,8	
PA1843	*metH*	methionine synthase		-6,0	
PA1927	*metE*	methionine synthase	-13,4	-13,7	
PA2442	*gcvT2*	glycine cleavage system protein T2			4,9
PA2444	*glyA2*	serine hydroxymethyltransferase		5,8	
PA2444	*glyA2*	serine hydroxymethyltransferase			27,8
PA2445	*gcvP2*	glycine cleavage system protein P2			23,1
PA2446	*gcvH2*	glycine cleavage system protein H2			16,2
PA4602	*glyA3*	serine hydroxymethyltransferase	-5,4	-12,3	
PA5415	*glyA1*	serine hydroxymethyltransferase		4,8	4,3
tRNAs:					
tRNA-Cys		PA2581.1	24,7	327,9	13,3
tRNA-Pro		PA2736.1/PA3031.1/ PA4541.2	6,1	27,9	
tRNA-Val		PA2775.1/PA3094.3/PA3262.2	4,2		
tRNA-Ser		PA2852.1/PA0905.1/ PA1013.1/PA2603.1	212,9	416,9	
tRNA-Pro		PA3031.1			4,6
tRNA-Asn		PA3139.1/PA4541.3		5,3	
tRNA-Arg		PA3368.1/PA4581.1/PA0905.2/PA0905.3/PA1796.1/PA0263.1		7,8	
tRNA-Leu		PA3824.1 /PA4937.1/ PA4937.2/ PA1796.3/PA2570.1/ PA4746.2	23,2	59,4	
tRNA-Thr		PA4277.1/PA4524.1/ PA5160.1		46,5	12,3
tRNA-Tyr		PA4277.3		5,1	4,2
tRNA-Met		PA4746.1/PA4673.1/ PA0574.1/PA0922.1		27,9	25,5
tRNA-Phe		PA5149.1	7,3	53,6	7,4

Continued:

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
Choline metabolism:					
PA3933		probable choline transporter	-5,2	-8,6	
PA5372	*betA*	choline dehydrogenase	-6,8	-12,4	
PA5373	*betB*	betaine aldehyde dehydrogenase	-10,3	-25,4	
PA5374	*betI*	transcriptional regulator BetI	-9,5	-33,0	
Fatty acid and phospholipid metabolism:					
PA4351		probable acyltransferase		31,4	31,4
PA0447	*gcdH*	glutaryl-CoA dehydrogenase		7,0	3,6
PA0228	*pcaF*	beta-ketoadipyl CoA thiolase PcaF	55,5		-54,9
Nucleotide biosynthesis and metabolism:					
PA0441	*dht*	dihydropyrimidinase	8,6		
PA0444		N-carbamoyl-beta-alanine amidohydrolase	22,3	6,5	-3,4
PA0849	*trxB2*	thioredoxin reductase 2	-8,4	-6,9	
PA4670	*prs*	ribose-phosphate pyrophosphokinase		-5,4	
PA5541	*pyrQ*	dihydroorotase	-47,2	-5,5	8,6

The *betAB* operon, which is regulated by *betI*, and a probable choline transporter PA3933 were upregulated under *ex vivo* conditions (A/EV ; ACh/EV). These genes have been reported to be involved in the utilization of choline as a carbon and nitrogen source (Son *et al.*, 2007). Moreover, *betB* as betaine aldehyde dehydrogenase is also involved in the production of glycine betaine, one of the major osmoprotectants of bacterial cells (Csonka and Hanson, 1991). Under *ex vivo* conditions these genes were upregulated compared to medium conditions, although high salt conditions are present in both environments, somehow the need for osmoprotectants in the CF airways might be increased. On the other hand phosphatidylcholine concentration could be limiting in the medium compared to the CF airways, although the medium contains egg yolk as source of lecithin.

The genes *trxB2*, *prs*, *pyrQ* seem to be more important under *ex vivo* conditions, since they are upregulated under *ex vivo* conditions. PyrQ is involved in *de novo* pyrimidine biosynthesis (Brichta *et al.*, 2004). In contrast, the genes *dht* encoding for a dihydropyrimidinase and PA0444 encoding for a N-carbamoyl-beta-alanine amidohydrolase were upregulated in ASM and ASM-Chelex. These genes are described to encode for proteins involved in the degradation of pyrimidine (Kim and West, 1991). This is indicating that more nucleotides are metabolismed in ASM and ASM-Chelex than under *ex vivo* conditions.

ASM and ASM-Chelex contain egg yolk, DNA, amino acids and mucin as nutrient sources to simulate the sputum composition of the CF airways. In the *ex vivo* sputum sample an upregulation of the genes *betAB, betI* and PA3933 indicates a strong prevalence of *P. aeruginosa* for choline as nutrient source. Nucleotides were hardly metabolised during *ex vivo* conditions. Methionine and SAM metabolism were induced under *ex vivo* conditions. That is very interesting since the most common auxotrophy reported for *P. aeruginosa* CF isolates is the methionine auxotrophy (Barth and Pitt, 1995; Barth and Pitt, 1996; Thomas *et al.*, 2000). Fatty acid and glycerol uptake and degradation might take place to the same degree *ex vivo* and under simulated conditions and therefore genes were not differently expressed.

Compared to the medium conditions less tRNAs were generated *ex vivo* that indicates that less protein biosynthesis took place. Moreover, amino acid transport, biosynthesis and metabolism were also decreased under *ex vivo* conditions.

2.2.13 Amino acid uptake from amino acid medium

Amino acid uptake was analysed. For this reason amino acid medium (AAM) was used, which reflects the amino acid composition of ASM but without DNA, mucin and egg yolk for easier analysis of the metabolic footprint. *P. aeruginosa* PAO1 was grown aerobically in AAM (Fig. 15). Interestingly, the amino acids glutamine and asparagine were consumed first. After 6 h of growth, only concentrations ≤ 30 µM of both amino acids remained. Palmer *et al.* (2007) did not detect these amino acids in their sputum samples and, therefore, did not include glutamine and asparagine in synthetic CF sputum medium (SCFM). The reason behind this fact might be the preferred uptake of glutamine and asparagine by *P. aeruginosa* as carbon and nitrogen source. The amino group of glutamine can be transferred via transaminase reaction to an α-keto acid. Furthermore, glutamine and asparagine can be easily used as energy source, if converted to glutamate and channelled via TCA cycle. Both amino acids contain one atom of nitrogen as an amide and another atom of nitrogen as an amine and are therefore perfect nitrogen sources for the cell for the synthesis of nucleotides, amino acids, amino-sugars and vitamins.

Aspartate, glutamate, alanine and arginine, were taken up to concentrations of ≤ 100 µM after 14 h of growth of PAO1 in AAM. This is according to the study performed by Behrends *et al. (*2009), which used NMR for time-resolved metabolic footprinting of metabolites in SCFM. They did not detect glutamine and asparagine, since it is not included in SCFM. Nonetheless, the fitted t_{50} values of the metabolites, which are the summarized uptake characteristics, are available for comparison (Behrends *et al.*, 2009). The t_{50} values show exactly the same order of uptake for the four amino acids. Only the time points vary between the studies due to different presentation. Most likely due to different methods employed, the uptake of the next four amino acids threonine, glycine, serine and lysine is different between the studies. Threonine, glycine, serine and lysine were consumed between 14 h – 24 h to concentrations ≤ 20 µM. Serine and glycine were also taken up in the study of Behrends *et al.* (2009) after the uptake of the first four amino acids. Leucine and isoleucine remained still at concentrations ≥ 100 µM after 24 h of *P. aeruginosa* PAO1 in AAM.

Ornithine concentration in the medium increased from 30 µM up to 80 µM -100 µM after 6 h and remained there till 14 h. Ornithine remained at ≥ 70 µM after 24 h. Ornithin is a degradation product of amino acids and of the amino acid catabolism, which is probably secreted by *P. aeruginosa*.

48 % of histidine, 41% of phenylalanine and 32 % of valine were taken up after 24 h. Only 10% of methionine and only 4% of tyrosine were consumed in the same time. Valine and methionine are also consumed very late in SCFM. Another interesting fact is that Behrends *et al.* (2009) did not detect uptake of methionine in LB medium.

Very interesting is also the correlation between growth behaviour and uptake. After 7 h with the depletion of glutamine and asparagine the culture entered early stationary phase. Afterwards only a slight increase in OD_{578} was detected. The growth behaviour also reflects the uptake and depletion of certain amino acids.

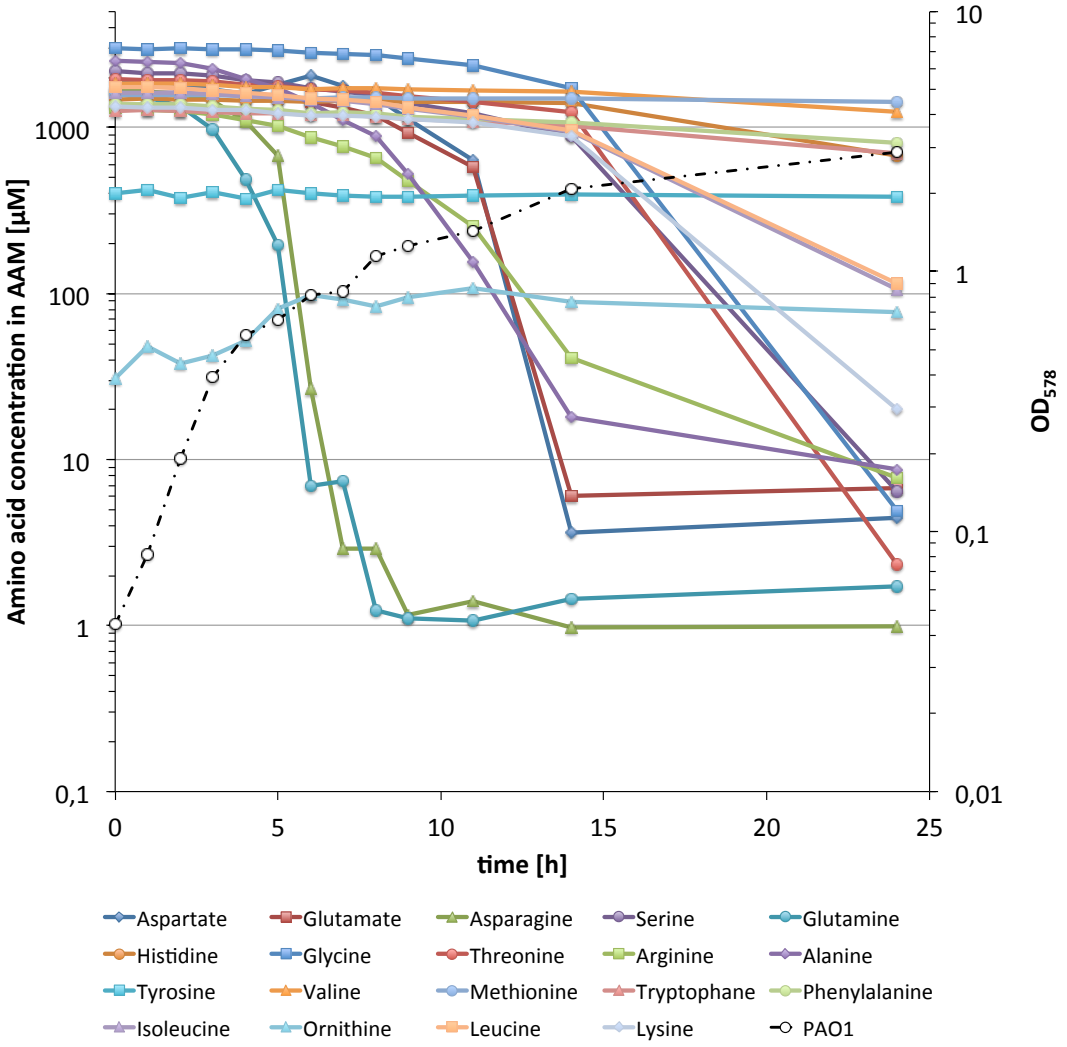


Fig. 15: Amino acid concentrations during growth of *P. aeruginosa* PAO1 in amino acid medium (AAM)

P. aeruginosa PAO1 (black dashed line, open circles) was grown in AAM for 24 h and medium samples were taken to determine the amino acid concentration in the AAM and OD_{578} of the culture was measured. The concentration over time of 18 of 20 the proteinogenic amino acids were measured, except for cysteine and proline, which were not detected in this approach. In addition ornithine formation was also determined.

Therefore, in media containing several nutrient sources expression and translation of transporter systems and catabolic pathways need to be regulated. Approximately 300 known or predicted nutrient uptake systems are found in the genomes of the Pseudomonas species (Tamber and Hancock, 2003). Some of them are also regulated in ASM as we have seen previously (Table 7).

Uptake requires energy and also catabolic pathways require certain amount of energy. As a result, the expressed pathways are tightly regulated, as we can observe in the uptake pattern. Not all amino acids were taken up at the same time and in the same amount. For example ArgR controls the aerobic catabolism of arginine (Park *et al.*, 1997), but also the level of OprD. OprD is a porine for basic amino acids (Ochs *et al.*, 1999) and moreover a serine transporter (Lu *et*

al., 2004). Furthermore Ochs *et al.* (1999) reported that *oprD* was induced by glutamate and alanine as sole carbon and nitrogen source. This is also reflected in the uptake pattern (Fig. 14) arginine, glutamate and alanine are taken up together and serine is consumed later.

2.2.14 Motility is reduced in ASM and ASM-Chelex compared to *ex vivo* sputum samples

The chemotaxis proteins PA0177 and PA1608 were induced in the *ex vivo* sputum sample and were therefore downregulated in ASM and ASM-Chelex (Table 8). PA0177 is 78% similar to chemotaxis protein CheW of *E. coli* and probably involved in purine detection. PA1608 is a probable chemotaxis transducer. The other probable chemotaxis transducer PA2573, PA4290 and PA4915 were upregulated in ASM and ASM-Chelex.

In contrast, to the predicted biofim and microcolony growth style during *in vivo* conditions the genes encoding for the flagella *flgC* (PA1078) and *flgE* (PA1080) were detected upregulated under *ex vivo* conditions (A/EV ; ACh/EV) (Table 8), since the genes were downregulated in ASM and ASM-Chelex. Both genes are predicted to be part of the *flgBCDE* operon. Under *ex vivo* conditions also the predicted *flgFGHI* operon was upregulated. Amiel *et al.* (2010) showed that the loss of flagella function and not the loss of the flagella apparatus itself is the critical factor associated with resistance to nonopsonic phagocytosis. Thereby, explaining the observed advantage of motility loss associated with higher bacterial burden in the lung. Therefore, the flagella might be still present on the clinical isolates CFCZ and CF-1 – 4 but not necessarily functional. The functionality of the flagella of both strains has to be verified.

On the other hand Roger *et al.* (2004) and Singh *et al.* (2002) reported that lactoferrin and conalbumin inhibited the biofilm formation of *P. aeruginosa*. They assumed that iron chelation led to increased surface motility by twitching motility and thereby inhibiting biofilm formation (Singh *et al.*, 2002).

An induction of the type IV pili genes *pilW* and *pilX* was observed in ASM-Chelex vs. *ex vivo* and ASM-Chelex vs. ASM conditions. Both proteins are located in the periplasm and belong to the minor pilins, which are the base for PilA (Giltner *et al.*, 2010; Mattick, 2002). Type IV pili mediate both attachment to host epithelial cells and flagella-independent twitching motility (Alm *et al.*, 1996). But type IV pili were not induced under *ex vivo* conditions.

Upregulation of *tpbA* a tyrosine phosphatase was observed under *ex vivo* conditions (A/EV ; ACh/EV). TpbA upregulation results in increased swimming, swarming and decreased attachment (Ueda and Wood, 2009; Yang *et al.*, 2010). All this indicates a planktonic life-style and less exopolysaccharide production under *ex vivo* conditions and biofilm formation under ASM and ASM-Chelex conditions. PA3885 protein is a negative regulator of c-di-GMP production. Uedo and Woods (2009) also showed a connection to LasR, which activates *tpbA* expression. Thereby, biofilm structure is linked to environment and cell density.

Motility and chemotaxis recognition were detected as important factors *in vivo*. Unexpectedly fagella genes seem to be expressed under *ex vivo* conditions indicating plantonic growth style. Type IV pili were repressed or decreased in expression under *ex vivo* conditions. One can speculate about the reasons for the planktonic life-style.

Table 8: Fold change of gene expression of selected motility genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 – 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A).

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
Chemotaxis:					
PA0177		probable purine-binding chemotaxis protein			-3,7
PA1608		probable chemotaxis transducer	-5,1	-5,1	
PA2573		probable chemotaxis transducer		4,9	
PA4290		probable chemotaxis transducer	10,2		
PA4915		probable chemotaxis transducer	6,3	6,7	
Motility and attachment:					
PA1077	*flgB*	flagellar basal-body rod protein FlgB		-6,8	
PA1078	*flgC*	flagellar basal-body rod protein FlgC	-5,9	-20,2	-3,4
PA1079	*flgD*	flagellar basal-body rod modification protein FlgD	-4,8	-11,7	
PA1080	*flgE*	flagellar hook protein FlgE	-7,4	-35,6	-4,8
PA1081	*flgF*	flagellar basal-body rod protein FlgF	-6,9	-8,2	
PA1082	*flgG*	flagellar basal-body rod protein FlgG		-7,5	
PA1083	*flgH*	flagellar L-ring protein precursor FlgH		-5,2	
PA1084	*flgI*	flagellar P-ring protein precursor FlgI		-6,7	
PA3885	*tpbA*	protein tyrosine phosphatase TpbA	-5,7	-7,0	
PA4552	*pilW*	type 4 fimbrial biogenesis protein PilW		5,7	4,4
PA4553	*pilX*	type 4 fimbrial biogenesis protein PilX		5,3	5,1
Pae_flgK	*flgK*	flagellar hook protein		-6,2	
M57501	*flaA*	Pseudomonas aeruginosa flagellin (flaA) gene	13,6	5,7	

2.2.15 Secretion and virulence factors

Not many virulence factors were expressed differently in ASM vs. *ex vivo*, ASM-Chelex vs. *ex vivo* and ASM-Chelex vs. ASM. That might be due to the fact that the conditions in ASM and ASM-Chelex were very similar to the *ex vivo* conditions and thereby to the *in vivo* conditions. For example, genes involved in alginate, cyanide, phospholipase C, protease and rhamnolipid production were not differently expressed compared to *ex vivo* sputum samples.

The expression of these virulence factors is also partly regulated by quorum sensing. The N-acylhomoserine lactone (AHLs) dependent quorum sensing systems LasI/LasR and RhlI/RhlR must be similarly regulated under the investigated conditions, since they show no difference in expression. Interestingly, the only difference in expression was observed for PQS system (Table 4), probably due to excess of aromatic amino acids in ASM and ASM-Chelex and the probably high PQS concentrations in the CF sputum compared to *ex vivo* (Palmer *et al.*, 2005). Several studies describe the loss of virulence factors during chronic CF infection (Barth and Pitt, 1995; D'Argenio *et al.*, 2007; Smith *et al.*, 2006).

In contrast, the upregulation of the type III secretion system (T3SS) genes and associated genes PA1698 - PA1725 (Table 9) was observed under *ex vivo* conditions.

Furthermore, the expression of the effector genes *exoS*, encoding for a ADP-ribosyltransferase and Rho GTPase-activating protein (GAP) and *exoT* gene, encoding for ADP-ribosyltransferase and Rho GAP (Yahr *et al.*, 1997) was upregulated under *ex vivo* conditions. The carriage of the *exoS* gene is more prevalent among *P. aeruginosa* clinical isolates (Kulasekara *et al.*, 2006). ExoS and ExoT confer anti-phagocytic abilities to *P. aeruginosa* mostly through their action on

the actin cytoskeleton (Bleves *et al.*, 2010). T3SS is an important factor during *in vivo* infection conditions. T3SS expression was detected upregulated under *ex vivo* conditions.

Table 9: Fold change of gene expression of selected virulence associated genes in ASM, ASM-Chelex and *ex vivo* sputum samples

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 – 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes during growth in ASM compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV), ASM-Chelex compared to ASM (ACh / A).

Gene ID	Gene name	Function	FC A / EV	FC ACh / EV	FC ACh / A
Secreted factors (toxins, enzymes, alginate):					
PA0044	*exoT*	exoenzyme T	-13,5	-13,2	
PA0085	*hcp1*	Hcp1			-7,0
PA1707	*pcrH*	regulatory protein PcrH	-9,0	-10,8	
PA3841	*exoS*	exoenzyme S	-7,7	-10,7	
PA3842		probable chaperone	-13,9	-14,3	
Protein secretion/export apparatus:					
PA1698	*popN*	Type III secretion outer membrane protein PopN precursor		-4,5	
PA1699		conserved hypothetical protein in type III secretion	-7,5	-7,5	
PA1700		conserved hypothetical protein in type III secretion	-9,0	-10,3	
PA1701		conserved hypothetical protein in type III secretion	-9,5	-9,5	
PA1702		conserved hypothetical protein in type III secretion	-18,9	-18,5	
PA1706	*pcrV*	type III secretion protein PcrV	-9,3	-9,4	
PA1707	*pcrH*	regulatory protein PcrH	-9,0	-10,8	
PA1708	*popB*	translocator protein PopB	-15,7	-16,1	
PA1710	*exsC*	ExsC, exoenzyme S synthesis protein C precursor.	-4,6	-6,2	
PA1711	*exsE*	ExsE	-8,5	-19,2	
PA1712	*exsB*	exoenzyme S synthesis protein B	-16,3	-20,3	
PA1713	*exsA*	transcriptional regulator ExsA	-5,6	-5,3	
PA1714	*exsD*	ExsD	-9,4	-12,7	
PA1715	*pscB*	type III export apparatus protein	-16,2	-16,2	
PA1716	*pscC*	Type III secretion outer membrane protein PscC precursor	-12,7	-14,7	
PA1717	*pscD*	type III export protein PscD	-7,6	-8,1	
PA1718	*pscE*	type III export protein PscE	-12,6	-12,6	
PA1722	*pscI*	type III export protein PscI	-7,2	-7,2	
Adaptation and protection:					
PA4110	*ampC*	beta-lactamase precursor	-48,3	-33,7	
Exopolysaccharide formation:					
PA2231	*pslA*	PslA exopolysaccharide biosynthesis	4,1		
PA2232	*pslB*	PslB exopolysaccharide biosynthesis	3,8		

In vivo, T3SS functioning is triggered by the contact with the eukaryotic cell, which leads to effector injection (Bleves *et al.*, 2010; Hauser, 2009). Moreover, T3SS transcription is induced by calcium limiting conditions (Bleves *et al.*, 2010). The presence of epithelial cells might trigger the induction of T3SS under *in vivo* conditions (Hueck, 1998; Miller, 2002; Rietsch *et al.*, 2004). The in ASM and ASM-Chelex observed repression is probably due to the lack of epithelial cell contact, as T3SS genes are expressed under *ex vivo* conditions.

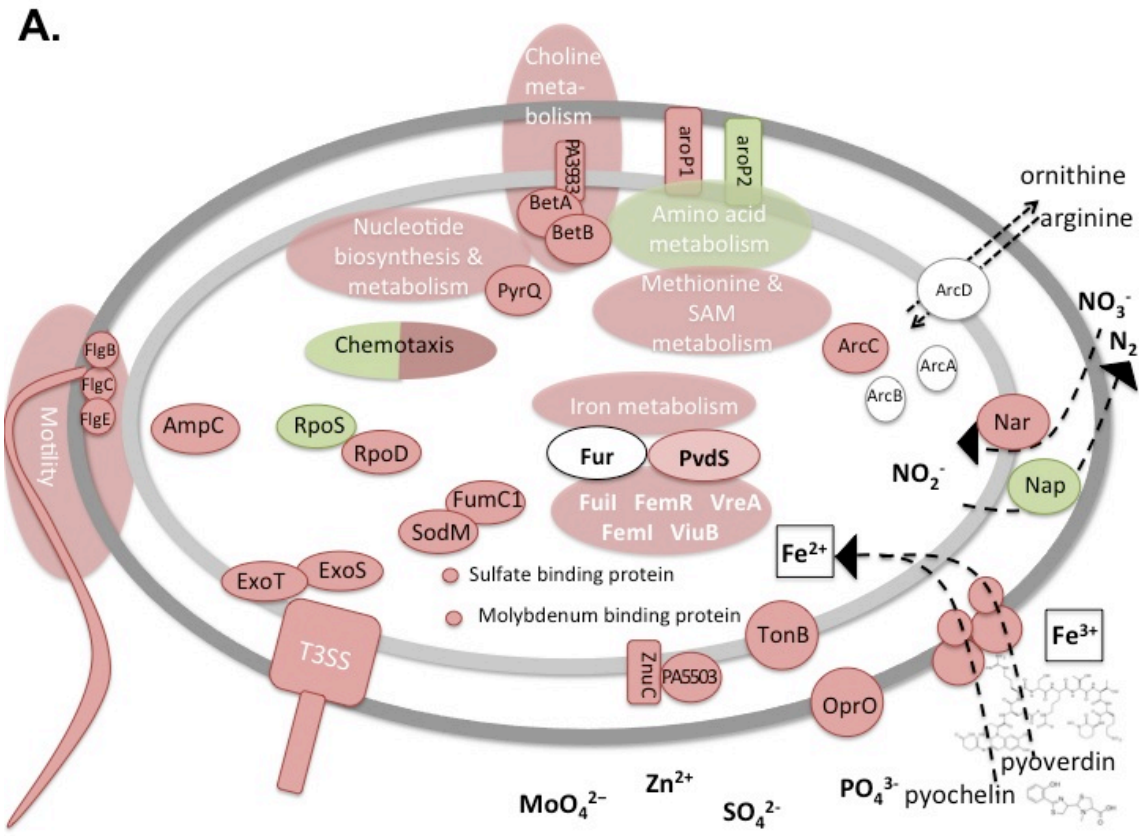
Interestingly, the upregulation of *pslAB* expression for the biosynthesis of the exopolysaccharide in ASM vs. *ex vivo* was observed. Both genes are required for Psl synthesis and furthermore *pslA* is necessary for solid surface attachment (Byrd *et al.*, 2009). A probable glycosyl transferase is encoded by *pslA*, whereas *pslB* encodes for a phosphomannose isomerase/GDP-D-mannose pyrophosporylase.

2.3 Conclusions

This study investigated how the growth conditions in the CF airways affect the infecting and persisting *P. aeruginosa* strains and how well these growth conditions can be simulated using ASM and ASM-Chelex. The global analysis of *P. aeruginosa* gene expression under simulated respiratory tract conditions *in vitro* in ASM and under *ex vivo* CF lung infection conditions is reported. Besides, Son *et al.* (2007) and the recently published *ex vivo* CF lung infection transcriptome data (Bielecki *et al.*, 2013) this is one of the few studies presenting *ex vivo* sputum sample transcriptome data. Moreover, this study is the first to compare the *ex vivo* expression results to simulated respiratory tract conditions in the model medium ASM by Sriramulu *et al.* (2005).

Iron limitation was identified as one of the crucial stress factors in the CF airways *in vivo.* As a result increased iron limitation was introduced in ASM and ASM-Chelex obtained, but the level of limitation has not reached the *ex vivo* level.

Two clonally distinct isolates of two independent CF patients were analysed. The transcriptional profile of theses strains was compared under *ex vivo* conditions to ASM and ASM-Chelex. A graphical summary of the findings is shown in Fig. 16. The model represents the transcriptional response of the clinical *P. aeruginosa* isolates CFCZ and CF1-4 under *ex vivo* vs. ASM and ASM-Chelex conditions (Fig. 16 A.), ASM vs. *ex vivo* conditions (Fig. 16 B.) and under ASM-Chelex vs. *ex vivo* conditions (Fig. 16 C.). The pathways and specific genes mentioned in the results and discussion section are reported. Furthermore, the up- (red) and downregulation (green) and stable (white) expressions of the genes encoding for the depicted proteins is shown. The genes and functional groups displayed are functionally classified according to "Pseudomonas Genome Database" (Winsor *et al.*, 2011).



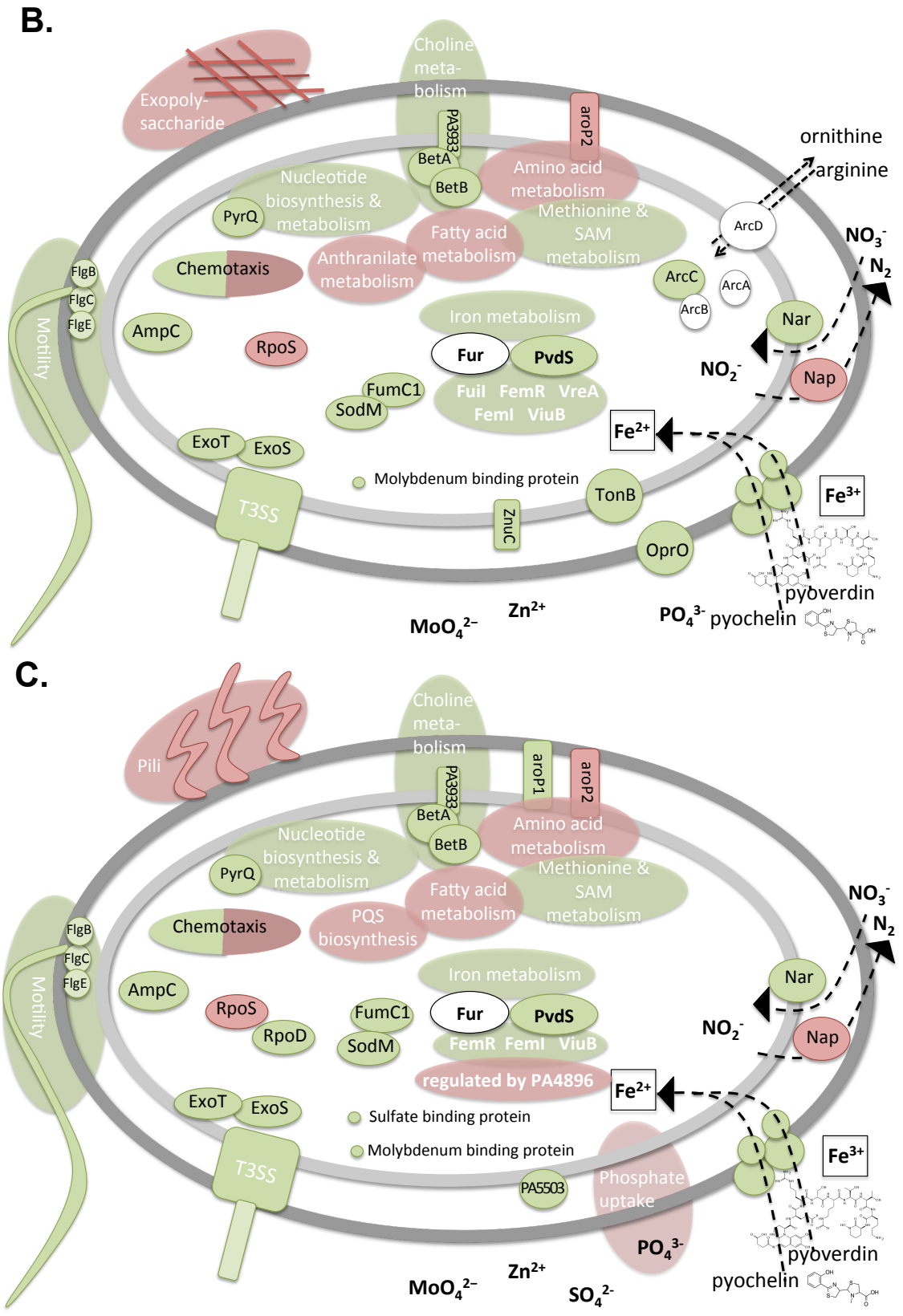


Fig. 16: Model of genes regulated in CF1-4 and CFCZ under *ex vivo* conditions, in ASM and ASM-Chelex

Model of genes regulated in CF1-4 and CFCZ under *ex vivo* conditions vs. ASM / ASM-Chelex (A), under ASM vs. ex vivo conditions (B) and under ASM-Chelex vs. ex vivo conditions (C). Colours

indicate the up- (red) and downregulation (green) and stable (white) expressions of the genes encoding for the depicted proteins.

The most interesting and important findings are discussed below.

Development of ASM-Chelex

The comparison of the transcriptional profile of ASM vs. *ex vivo* showed that ASM contained higher amounts of iron than available under *ex vivo* conditions (2.2.3 and Table 2). Initial testing using qRT-PCR revealed induction of iron limitation marker genes in ASM-Chelex by the addition of Chelex (Fig. 7). In accordance with the qRT-PCR the stronger induced marker genes were also identified by the conducted microarray in ASM-Chelex (Table 3).

Is ASM-Chelex simulating iron limitation more closely than ASM?

The scatter plots and correlation values indicate no higher correlation rate between ASM-Chelex vs. *ex vivo* than ASM vs. *ex vivo* (Fig. 8). As iron limitation is only influencing a relatively small number of genes, no change of correlation values is observed.

The comparison of the transcriptional profiles of ASM-Chelex vs. *ex vivo*, ASM vs. *ex vivo* and ASM-Chelex vs. ASM revealed that the expression level of several iron-regulated genes in ASM-Chelex was closer to *ex vivo* conditions compared to ASM (Table 3). Especially the comparison of ASM-Chelex vs. ASM showed the significant upregulation of pyochelin biosynthesis genes in ASM-Chelex. However, many of observed other changes in expression were only minor.

In general, iron limitation is increased in ASM-Chelex compared to ASM, although the degree of iron limitation is not as high as expected.

Iron limitation might not be as strong as under *ex vivo* conditions since the response to iron limitation might be intensified in stationary phase, when the *P. aeruginosa* strains are completely depleted of iron. The *P. aeruginosa* strains grown in ASM and ASM-Chelex are probably “younger cultures” than the strains isolated from the *ex vivo* samples (see below). Moreover, host proteins chelating iron with very high affinity such as ferritin, lactoferrin, transferrin or heme are present under *in vivo* conditions increasing the iron limitation.

Expression of non-iron regulated genes in *P. aeruginosa* in ASM-Chelex

Unexpectedly, the expression of other non-iron regulated genes was more heterogeneously in ASM-Chelex vs. *ex vivo*. The expression of anthranilate metabolism genes was *ex vivo*-like in ASM-Chelex (Table 4). In contrast the PQS biosynthesis genes were detected upregulated in ASM vs. *ex vivo* and ASM-Chelex vs. *ex vivo*, probably due to excess of aromatic amino acids in ASM and ASM-Chelex and the probably high PQS concentrations in the CF sputum compared to *ex vivo* (Palmer *et al.*, 2005).

Moreover, an unexpected induction of phosphate starvation gene response was observed in ASM-Chelex vs. *ex vivo* (Table 5). The Chelex treatment did probably not only remove the iron ions but also decreased the amount of available phosphate. One can discuss if the metal ions in

ASM-Chelex, which are chelated by the Chelex resin, were acting in combination with Chelex probably as very efficient phosphate scavengers (Wu *et al.*, 2007; Zhao and Sengupta, 1998).
The gene expression in ASM and ASM-Chelex indicates that the *P. aeruginosa* strains were metabolically active. Especially, the induction of the t-RNAs genes and amino acid transporter genes confirmed this observation (Table 7).
The growth conditions were aerobic to microaerobic in ASM and ASM-Chelex, which was also indicated by the expression of the different oxidases with different oxygen affinities. The *cox* operon coding for the aa_3-type low-affinity oxidase is preferably expressed in the stationary phase in an aerobic environment (Alvarez-Ortega and Harwood, 2007; Schuster *et al.*, 2004). The cbb_3-type 2 oxidase operon, which is positively regulated by Anr and RoxSR and expressed at low oxygen levels (Alvarez-Ortega and Harwood, 2007; Kawakami *et al.*, 2010), was upregulated in ASM-Chelex as well, indicating microaerobic conditions.

Physiology of *P. aeruginosa* under *ex vivo* conditions

Unexpectedly, the strains grown under *ex vivo* conditions were metabolically more inactive than strains grown in ASM or ASM-Chelex for 24 h. Strains under both, medium and *ex vivo* conditions, reached stationary phase. Under *ex vivo* condtions the *P. aeruginosa* strains CFCZ and CF1-4 must have been in a different maybe much later state of stationary phase compared to the same strains grown in ASM or ASM-Chelex.
During *ex vivo* conditions *P. aeruginosa* strains were growing and surviving by denitrification (Table 6) under microaerobic to anaerobic conditions. This is in accordance with literature, which describes the microaerobic to anaerobic conditions for the CF lung and the possibility of denitrification (Moreau-Marquis *et al.*, 2008; Worlitzsch *et al.*, 2002; Zumft, 1997). The conditions *in vivo* might supplement more nitrate than ASM or ASM-Chelex, since physiological nitrate and nitrite concentrations in patient's sputum vary from 73 µM to 792 µM (Grasemann *et al.*, 1998; Hassett, 1996; Linnane *et al.*, 1998; Palmer *et al.*, 2007b). This might allow the very slow growth indicated by gene expression for the strains CFCZ and CF1-4.
Although the *P. aeruginosa* strains are metabolically very inactive the gene *aroP1* encoding for a transporter, was induced under *ex vivo* conditions and not in ASM and ASM-Chelex (Table 7). Not much is know about the AroP1 transporter. It is annotated to transport aromatic amino acids (Winsor *et al.*, 2011). Therefore, it would be interesting to investigate, which substances are transported during *ex vivo* conditions.
The induction of methionine and SAM metabolism under *ex vivo* conditions (Table 7) correlates with the observed late uptake of methionine during growth in AAM (Fig. 15). It would be interesting to investigate, why the greater amount of methionine uptake is only taking place during stationary phase. Hence methionine is needed for metabolism during all growth phases. Moreover, methionine auxotrophy is reported as the most common amino acid auxotrophy in *P. aeruginosa* CF isolates (Barth and Pitt, 1995; Barth and Pitt, 1996). The investigated strain CFCZ is a methionine auxotrophic strain (Chapter 3), whereas the strains CF1-4 are prototrophic strains. Therefore, especially the CFCZ strains might have been facing methionine limitation under *ex vivo* conditions. The methionine auxotrophic strains seem to possess a selection advantage of a yet unknown kind. By using these patient isolates` the study is

reflecting common CF phenotypes and is presenting their transcriptomic adaptations for the first time.

As described above iron limitation is the major stress condition for *P. aeruginosa* in the CF lung infection. Therefore, Fur - the ferric uptake regulator - as major regulator of iron acquisition, uptake and storage induces a complex regulatory network to obtain iron during CF infection. The majority of iron seems to be bound to heme during *ex vivo* conditions, since the gene *hasAp*, which is encoding for the heme acquisition protein, was the gene with the highest induction under *ex vivo* conditions (Table 3). One can assume that the transcriptional answer to iron limitation reflects the more general answer of *P. aeruginosa* towards infection.

Unexpectedly, the genes encoding for the flagella biosynthesis were found induced under *ex vivo* conditions (Table 8). This is in contrast to literature, which describes the loss of flagella during chronic CF infection (Amiel *et al.*, 2010; Mahenthiralingam *et al.*, 1994). Amiel *et al.* (2010) showed that the loss of flagella function and not the loss of the flagella apparatus itself is the critical factor associated with resistance to nonopsonic phagocytosis. Therefore, the flagella might still be present on the clinical isolates CFCZ and CF-1 – 4 but not necessarily functional. Moreover the *P. aeruginosa* strains seem to represent more a planktonic than a biofilm life-style under *ex vivo* conditions (Table 8). This is also a very interesting new observation in contrast to common literature (Costerton *et al.*, 1999; Govan and Nelson, 1993; Lam *et al.*, 1980)

The genes encoding for the T3SS system were also detected upregulated under *ex vivo* conditions. This is also a new an unexpected finding. Vfr is described to regulate T3SS system expression, while downregulating flagella biosynthesis at the same time (Dasgupta *et al.*, 2002; Wolfgang *et al.*, 2003). On can assume that the regulation might be altered in the clinical *P. aeruginosa* strains CFCZ and CF-1- 4.

Implications of metabolic inactivity during *ex vivo* conditions

Due to the metabolic inactivity the *P. aeruginosa* strains might be protected from antibiotics. Active uptake of antibiotics is prevented by the reduced expression of transporters. Many life-processes, which are targeted by antibiotics such as cell-wall biosynthesis, protein biosynthesis and DNA and RNA replication might not be taking place or only to a very limited degree. This is indicating why a *P. aeruginosa* infection is so difficult to treat once this state of metabolic inactivity is reached. Interestingly, the beta-lactamase precursor gene *ampC*, which was induced under *ex vivo* conditions, indicates that the patients have been treated with antibiotics prior to sampling or that the strains are constitutively expressing the *ampC* gene and are therefore resistant to β-lactam and cephalosporin antibiotics.

It is not known if there are substances present in the sputum leading to the very inactivated metabolism. On the other hand nutrients limitation and reduced availability of external electron acceptors or sources for substrate-phosphorylation might also lead to the metabolic inactivity. Therefore, it would be interesting to analyse the sputum composition as well as to perform a transcriptional analysis in the future. Moreover, antimicrobial peptides and other microorganisms might be present in the sputum influencing the metabolic activity of the *P. aeruginosa* strains.

With ASM-Chelex a powerful medium to investigate *P. aeruginosa* under simulated respiratory tract conditions was obtained, which is even closer to *in vivo* conditions than ASM, although little modifications have to be implemented such as increased phosphate addition to overcome the phosphate limitation, induced by Chelex to get closer to *ex vivo* conditions. Moreover, the incubation times of the *P. aeruginosa* strains have to be optimized.

2.4 Material and Methods

2.4.1 Bacterial strains and growth conditions

All bacterial strains used in this study are shown in Table 10. *P. aeruginosa* strains were grown in artificial sputum medium (ASM) and amino acid medium (AAM) to simulate the conditions of the cystic fibrosis lung.

Cultures were inoculated at OD_{578} of 0.05 and shaken at 200 rpm at 37°C, if not indicated otherwise. ASM (pH 6.9) consists of 86 mM NaCl, 29 mM KCl, 20 mM Hepes, 15 µM diethylene triamine pentaacetic acid (DTPA; Fluka), 39.7 mM amino acids [2.7 mM alanine, 1.6 mM glutamate, 1.8 mM isoleucine, 1.2 mM tryptophan, 1.8 mM leucine, 1.8 mM aspartate, 2.1 mM proline, 1.6 mM lysine, 2.3 mM serine, 1.6 mM methionine, 1.5 mM phenylalanine, 1.3 mM tyrosine, 2.6 mM valine, 2.5 mM threonine, 2.5 mM cysteine, 2.3 mM asparagine, 2.1 mM glutamine, 1.7 mM arginine, 3.3 mM glycine, 1.5 mM histidine (Sigma)], 4 g/l DNA, (Serva) 5 g/l mucin (Sigma) and 0.5 % sterile egg yolk (Fluka) (Sriramulu *et al.*, 2005). The pH was adjusted with HCl and NaOH. DNA and amino acids were sterilised by filtration. Nitrate and nitrite concentrations of 260 µM and 130 µM were supplemented, respectively. For solid medium 15 g/l agar were used.

AAM (pH 6.9) contains 86 mM NaCl, 29 mM KCl, 20 mM Hepes, 15 µM DTPA, 1.75 mM $CaCl_2$, 0.61 mM $MgCl_2$, 0.271 mM $MgSO_4$ and 2.55 mM Na_2HPO_4/ NaH_2PO_4 (pH 6.9) and 39.7 mM amino acids (as described for ASM). The pH was adjusted with HCl and NaOH.

For growth experiments in ASM an over night culture in LB medium was prepared. For modified iron-free ASM the medium was incubated for 24 h with 3 and 4 mg/ml conalbumin (Sigma) or 0.5 and 1 mg/ml lactoferrin (Sigma), respectively. For ASM-Chelex the Chelex resin (Biorad) was autoclaved separately in MilliQ with 17 mM NaCl, 20 mM Hepes (pH 6.8). 500 µl Chelex solution (0.1 g/ml) was mixed with 500 µl double concentrated ASM.

Cultures were grown in 1 ml medium in a 24 well plate (Thermo Fisher Scientific) and sealed with sealing film Breath-easy (Roth) for gas exchange and shaken at 300 rpm (Thermo Shaker PST-60 HL-4, Lab4You) at 37°C. Bacteria were harvested after 24 h.

2.4.2 Determination of amino acid uptake from AAM

P. aeruginosa PAO1 was grown in AAM for the determination of amino acid uptake. During growth OD_{578} was determined routinely. Amino acids were quantified by HPLC (Agilent), as described previously (Kromer *et al.*, 2005). Culture supernatants were sterilised by filtration and diluted 1:10 on a microbalance (ALJ 160-4NM; Kern & Sohn) with an aqueous standard solution of α-aminobutyrate. After automated pre-column derivatization using O-phthaldialdehyde (OPA), detection was performed by fluorescence detection (340 nm excitation, 540 nm emission, Agilent).

Separation of culture supernatants and cell extracts was carried out on a reversed-phase (RP) column (Gemini 5 µ C18 110 Å, 150 × 4.6 mm, Phenomenex) equipped with a pre-column (Gemini C18, MAX-RP, 4 mm × 3 mm, Phenomenex) and a flow rate of 1 ml/min using 40 mM NaH_2PO_4 (eluent A, pH 7.8, adjusted with NaOH) as polar phase and a methanol/acetonitrile/water mixture (45:45:10, eluent B) and 40 °C.

2.4.3 RNA isolation and bacterial RNA enrichment

For RNA isolation 1 ml of bacterial culture grown in ASM was incubated with RNAprotect® Bacteria Reagent (Qiagen) according to the manufacturer's instructions. Samples were either processed directly or stored at - 80°C. RNA was isolated using RNeasy® Mini Kit (Qiagen). The RNA isolation was performed as described previously with minor modifications (Bielecki *et al.*, 2011). Bacteria were lysed using 400 µl TE buffer containing 1 mg/ml lysozyme with periodic vortexing every 2 min for 15 sec for 10 min. 700 µl RLT puffer containing 1% β-mercaptoethanol were added. Afterwards samples were centrifuged at maximal speed (14.100 x g) for 2 min. The same amount of ethanol was added to the samples. The samples were loaded to a spin column, washed with RW1 buffer afterwards DNA was digested using RNase-free DNase I. RNA was eluted in 50 µl and 30 µl of RNase-free water. Eluted RNA was treated a second time with DNase I to ensure that all traces of genomic DNA were removed. The isolated RNA was stored at – 80°C. The yield of the isolated RNA was measured by light absorption at 260 nm using a Nanodrop ND-1000-UV/VIS (Peqlab). Integrity and purity was checked by formaldehyde gel electrophoresis or by 2100 BioAnalyzer (Agilent Technologies).

2.4.4 RNA enrichment and amplification of cDNA for microarrays

Since the isolated RNA of the strains CFCZ and CF-1, CF-2, CF-3 and CF-4 should be compared to the *ex vivo* patient's samples, which contained both bacterial and eukaryotic RNA, the samples were enriched for bacterial RNA with the MicrobEnrich Kit® (Ambion), as described previously (Bielecki *et al.*, 2011).

P. aeruginosa GeneChip from Affymetrix requires 7,5 - 10 µg of RNA per microarray. It is not often possible to obtain these amounts from *ex vivo* patient's samples, therefore bacterial RNA amplification was used. This was performed using the MessageAmp Bacteria Kit® (Ambion). The first step was to treat the total enriched bacterial RNA with a polyadenylation polymerase to produce polyA tails. The single stranded cDNA was produced using reverse transcriptase and oligo dT primers. Afterwards a second strain cDNA was produced, and the double stranded cDNA served as a template for *in vitro* transcription using T7 RNA polymerase and T7 oligonucleotides. During the *in vitro* transcription reaction the modified nucleotides biotin-11-CTP (PerkinElmer Life Sciences) and biotin-16-UTP (Roche Applied Science) were used. As a result antisense biotinylated RNA ready to use for GeneChip® hybridization was obtained.

2.4.5 Microarray hybridisation and data normalisation

Amplified RNA is commonly used for eukaryotic microarrays, but the original procedure for *P. aeruginosa* Gene-Chip® (Affymetrix) was for single stranded terminally labelled cDNA. As described previously, no difference in results for microarray analysis were observed for the standard procedure (without amplification) RNA procession or amplified RNA (Francois *et al.*, 2007). The hybridization and washing steps were performed in the Affymetrix Array facility at Helmholtz Centre for Infection Research (HZI) Braunschweig (Dr. Robert Geffers). Since the RNA had been amplified, some changes were introduced to the original Affymetrix protocol, such as that the RNA was fragmented using 5 x fragmentation buffer instead of DNase I treatment. Hybridisation was performed as described previously (Bielecki *et al.*, 2011). Furthermore data normalisation and calculation of differential expression was performed with

software from Bioconductor microarray analysis suite (Gentleman *et al.*, 2004). Other aspects as quality analysis, the distribution, relative log expressions, normalised unscaled standard errors and expression values were computed, as described by Bielecki *et al.* (2011).

2.4.6 cDNA amplification for quantitative real-time polymerase chain reaction

Quantitative real-time PCR (qRT-PCR) was performed with SsoFast Evagreen Supermix (Bio-Rad) according to manufacturer's instructions. The PCR reactions were recorded with CFX96 Real-Time System (Bio-Rad). Cells were harvested and total RNA was prepared. cDNA was used as template for qRT-PCR and generated from 10-15 µg total RNA. 15 µg random primers and MilliQ water were added to the RNA to a final volume of 16 µl. Primer annealing was performed for 10 min at 70 °C. Afterwards 6 µl 5 x first strand buffer, 3 µl 100 mM DTT, 1 µl dNTPs (25 mM each) 20 U Superase*In were added and the reaction mixture was incubated for 2 min at 25 °C. Subsequent cDNA synthesis was carried out by addition of 600 U SuperScript II and incubation for 2 h at 42 °C. To remove RNA templates after reverse transcription, 10 µl 1 M NaOH and 10 µl 0.5 M EDTA solution (pH 8.0) were added. After 15 min at 65 °C 25 µl 1 M HEPES (pH 7.5) and 50 µl 3 M sodium acetate (pH 5.2) were used to neutralize the samples. cDNA was purified with QIAquick PCR Purification Kit according to manufacturer's instructions. For qRT-PCR 1 ng and 10 ng of cDNA were used as a template. All reactions were performed in triplicate, and no template and no reverse transcription controls were carried out in parallel. Standard qRT-PCR programs were carried out with an initial denaturation step at 98°C for 3.5 min, followed by 40 x repeated cycles of denaturation for 5 sec at 98°C, primer annealing for 15 sec at 59°C and elongation for 15 sec at 60°C. A plate read followed each cycle. Melting curves were generated by a final denaturation step for 10 sec at 98°C and recorded within the range of 65 - 98°C. Primer design was performed with Primer3Plus (Untergasser *et al.*, 2007) according to the following criteria: 100 - 150 bp product length, 18 - 30 bp primer length, 45 – 65 % GC content, 63 - 67 °C primer melting temperature and 65 - 85 °C product melting temperature. Table 11 lists the primers used for detection of genes associated with iron starvation genes in PAO1. We monitored *pvdA*, *pvdF*, *pvdS*, *fpvA*, *fptA* and *pchG* and used *acpP* as reference gene.

2.4.7 qRT-PCR data normalisation

Relative quantification was used to determine the ratio between the quantity of the target genes *pvdA*, *pvdF*, *pvdS*, *fpvA*, *fptA* and *pchG* in ASM and in the three modified putative iron-free ASMs (ASM-Conalbumin, ASM-Lactoferrin and ASM-Chelex) and the reference gene *acpP*. Several mathematical models have been formulated for the calculation of the expression of target genes compared to reference genes. Calculations are based on the comparison of distinct threshold cycle values (Ct) at a particular fluorescence level. We used the ΔΔCt method (Livak and Schmittgen, 2001). Therefore the obtained relative gene expression data were normalised against the reference gene *acpP*. ASM was used as reference sample, further referred to as calibrator.

First the ΔCt values for the ASM-Conalbumin, ASM-Lactoferrin and ASM-Chelex (1) and the calibrator sample (2) were calculated, as follows:

(1) $\Delta Ct_{treated} = Ct\ target_{treated} - CT\ reference_{treated}$

(2) $\Delta Ct_{calibrator} = Ct\ target_{calibrator} - CT\ reference_{calibrator}$

Then the $\Delta\Delta Ct$ value for each of the treated samples (3) was calculated.

(3) $\Delta\Delta Ct_{treated\ sample} = \Delta Ct_{treated\ sample} - \Delta Ct_{calibrator\ sample}$

Afterwards the target gene expression in each of the treated samples was related to the calibrator sample. The change in gene expression is $2^{-\Delta\Delta Ct}$, which is the relative quantity (RQ). Results are also be shown as fold difference $Log_2(RQ) = -\Delta\Delta Ct$.

Table 10: *Pseudomonas aeruginosa* strains

Bacterial strains	Genotype	Reference
PAO1	Wild type	(Dunn and Holloway, 1971)
CFCZ	Clinical cystic fibrosis isolate from patient A, Medizinische Hochschule Hannover	(Bielecki *et al.*, 2013)
CF-1	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
CF-2	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
CF-3	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
CF-4	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)

Table 11: Primers used for detection of transcript levels of *Pseudomonas aeruginosa* PAO1 iron starvation genes *pvdA*, *pvdF*, *pvdS*, *fpvA*, *fptA* and *pchG* during qRT-PCR

Gene	Primer name	Primer sequence (5' - 3')	Product size
pvdA	oAW083	GTCTTCTACCGCCAGAAAGTCTCC	147 bp
(PA2386)	oAW084	CGTCGTAGGTCTCTACGCTTAGCTC	
pvdF	oAW087	GGCCTATATCTGGTCCCTGAGAAAC	102 bp
(PA2396)	oAW088	AAGGACTCCAGTACCGACTTCATGT	
pvdS	oAW095	GATAACCGTACGATCCTGGTGAAGA	143 bp
(PA2426)	oAW096	AGGTAGCTGAGCTGTGCCTTGAAC	
fpvA	oAW091	GCTGGCTATACCCACAAGATCATTC	105 bp
(PA2398)	oAW092	GCCCTTGAACTTGTAGCTGGTGTAG	
fptA	oAW097	GTATGTCAGCTACGCCGAGGTCTAC	106 bp
(PA4221)	oAW098	CTTGATCCCGGTTTCGTAGGTCTT	
pchG	oAW101	CTCAACACCTTCTATCCGCAGTTG	135 bp
(PA4227)	oAW102	GATGTCCAGGGTGGCGAAAC	
acpP	oAS175	GAAGGAAGAAGAAGTCACCAACAGC	130 bp
(PA2966)	oAS176	TTCAGCTTTCTCGTCAGGGATTTC	

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3 Methionine auxotrophy - An adaptation strategy of *Pseudomonas aeruginosa* towards the cystic fibrosis lung

3.1 Introduction

Adaptive evolution due to genetic variation seems to be one of the key aspects of *Pseudomonas aeruginosa* survival in the challenging environment of the cystic fibrosis (CF) lung during chronic infection (D'Argenio *et al.*, 2007; Mena *et al.*, 2008; Smith *et al.*, 2006).

Host immune response and reoccurring antibiotic treatment create selective pressure. Oxygen limitation and the microaerobic to anaerobic conditions in the CF lung are also important factors shaping the *P. aeruginosa* phenotype (Alvarez-Ortega and Harwood, 2007; Worlitzsch *et al.*, 2002). This environment leads to the known phenotypes of mucoidy and the formation of microcolonies and biofilms (Boucher *et al.*, 1997; Costerton *et al.*, 1999; Govan and Deretic, 1996; Lam *et al.*, 1980).

Clinically relevant phenotypic changes are detectable, as *P. aeruginosa* colonizes the nutrient rich mucus of the CF airways, such as colony variation (von Gotz *et al.*, 2004), loss of motility (Amiel *et al.*, 2010; Mahenthiralingam *et al.*, 1994), modification of LPS (Hancock *et al.*, 1983), loss of quorum sensing (D'Argenio *et al.*, 2007; Smith *et al.*, 2006), reduced virulence (Luzar and Montie, 1985) and increased antibiotic resistance (Ciofu *et al.*, 2001; Wiegand *et al.*, 2008). It was shown that the mutator phenotype is widely spread among *P. aeruginosa* CF isolates and very important for adaptation (Ciofu *et al.*, 2005; Hogardt *et al.*, 2006; Oliver *et al.*, 2000).

Previous studies investigated the influence of nutrient availability and its direct influence on bacterial metabolism, on growth and virulence factor production (Palmer *et al.*, 2007a; Palmer *et al.*, 2005; Son *et al.*, 2007; Sriramulu *et al.*, 2005). In addition, it was frequently observed that some *P. aeruginosa* strains isolated from the CF lung were unable to synthesise particular growth factors (Taylor *et al.*, 1992). Strains were auxotrophic for certain nutrients. The most common source of auxotrophy was amino acids in *P. aeruginosa*. Most frequently methionine auxotrophic *P. aeruginosa* strains were isolated (Barth and Pitt, 1995; Barth and Pitt, 1996; Barth *et al.*, 1998; Thomas *et al.*, 2000). The question remains, which pathways are blocked in in the methionine auxotrophic *P. aeruginosa* strains.

Methionine and S-adenosylmethionine (SAM) metabolism are linked to one another (Fig. 25). Methionine and ATP are converted by S-adenosylmethionine synthetase MetK into SAM. SAM is the second most frequently used enzyme substrate after ATP (Fontecave *et al.*, 2004; Loenen, 2006). The majority of estimated 95 % of SAM is used for methylation reactions (Griffith, 1987). Methylation is one of the most ubiquitous chemical reactions in cellular metabolism (Stepkowski *et al.*, 2005). S-Adenosylhomocysteine (SAH) is the product of these reactions and a potent inhibitor of transmethylation reactions and therefore efficiently converted to homocysteine (Hcy) and adenosine (Ado) by S-adenosylhomocysteine hydrolase SahH. The methylation of macromolecules and small molecules is dependent on the intracellular SAM / SAH equilibrium. Recently, the intracellular SAM and SAH level for the *Escherichia coli* wild type strain MG1665 (OD_{600} of 1.62 ± 0.16) was determined with a ratio of 300:1 with 0.4 mM and 1.3 µM, respectively (Halliday *et al.*, 2010). Hcy can also be generated by MetZ from O-

succinyl-homoserine. The last step of methionine biosynthesis is the conversion of Hcy via either MetE or MetH to methionine. MetH is a cobalamine dependent and MetE a cobalamine independent methionine synthase. Both enzymes use the cofactor N^5-methyl-tetrahydrofolate (Me-THF) and N^5-methyl-tetrahydropteroyltriglutamate (Me-THP) as methyl-group donor, respectively. Methionine can be directly incorporated into biosynthesis or it can be converted to SAM as described.

Approximately 2-5% of cellular SAM is used for decarboxylation reactions e.g. for the production of polyamines or quorum sensing molecules synthesis such as N-acylhomoserine lactones (AHLs) or as source of 5´deoxyadenosyl radicals, which are used for the production of vitamins e.g. biotin and lipoate etc. (Griffith, 1987; Parveen and Cornell, 2011). These reactions yield 5´-methylthioadenosine (MTA) or 5´deoxyadenosine (5´dAdo), respectively.

Guan *et al.* (2011) propose that MTA is converted into 5´-methylthioinosine (MTI), which is further metabolised into hypoxanthine and 5´-methylthioribose-1-phosphate (MTR-1-P). MTR-1-P is further recycled into methionine by the gene products of *mtnA*, *mtnB*, *mtnC*, *mtnD* and *tyrB* (Heurlier *et al.*, 2006).

Interestingly, AHL biosynthesis is connected to the methionine and SAM metabolism. AHLs are synthesized of SAM and an intermediate of fatty acid biosynthesis by the AHL synthases of the LuxI family (Heurlier *et al.*, 2006). N-butyryl homoserine lactone (BHL) is synthesized by RhlI and N-3-oxododecanoyl homoserine lactone (odDHL) by LasI. BHL can diffuse freely (Wagner *et al.*, 2003; Williams and Camara, 2009). odDHL can diffuse but its transport is increased by *mexAB-oprM* and other efflux pumps.

Interestingly, several genes of methionine and SAM metabolism are described to be under positive quorum sensing control such as the genes encoding for the cleavage system *gcvT2H2P2*, *glyA1* hydroxy-methyltransferase, *pvdQ* and *metE* (Schuster *et al.*, 2003; Wagner *et al.*, 2003).

This study aimed at the identification of the genetic cause of methionine auxotrophy. To address this question 30 methionine auxotrophic *P. aeruginosa* CF stains from 15 different patients were isolated. Interestingly, a gene of the cofactor biosynthesis was identified as cause of methionine auxotrophy in the majority of strains. Unexpectedly, all methionine auxotrophic strains did not produce pyocyanin. To understand the underlying adaptation strategies several methionine metabolism gene deletion mutants in PAO1 were constructed. In accordance numerous PAO1 methionine metabolism gene deletion mutants showed reduced to abolished pyocyanin production. Methionine auxotrophy was identified as a new factor influencing pyocyanin biosynthesis in this study.

Moreover, the methionine auxotrophic strains and PAO1 methionine metabolism deletion mutants were phenotypically characterised. Another important factor during infection is the availability of iron. Iron was identified as influencing factor in the *P. aeruginosa* PAO1 cofactor biosynthesis deletion strain.

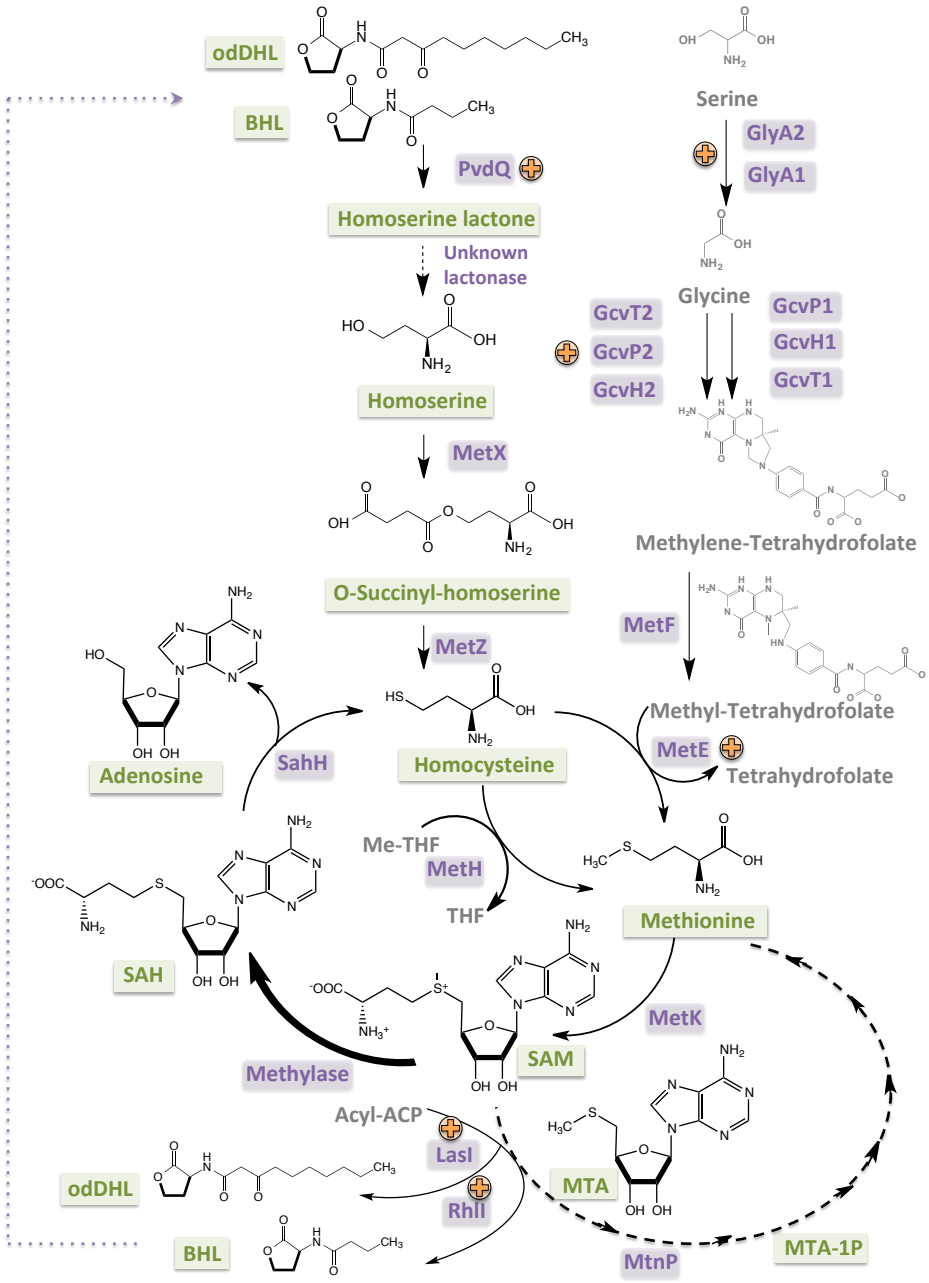


Fig. 17: Methionine metabolism and SAM recycling in *Pseudomonas aeruginosa*

Methionine and ATP are converted by S-adenosylmethionine synthetase MetK into SAM, phosphate and diphosphate. SAM is used for various metabolic reactions in the cell. If SAM is used for methylation the remaining SAH is hydrolysed by SahH into Ado and Hcy. Hcy can be generated out of O-succinyl-homoserine by MetZ. Hcy can be converted by either cobalamin-independent methionine synthase MetE or cobalamin-dependent methionine synthase MetH back into methionine. Both enzymes use the cofactor N^5-methyl-tetrahydrofolate (Me-THF) and N^5-methyl-tetrahydropteroyltriglutamate (Me-THP) as methyl-group donor, respectively (only THF is shown). MetF converts N^5,N^{10}-methylene-tetrahydrofolate to Me-THF or N^5,N^{10}-methylene-tetrahydropteroyltriglutamate to Me-THP. GlyA1 and GlyA2 are serine hydroxymethyl transferases, which convert serine into glycine and thereby produce N^5,N^{10}-methylene-tetrahydrofolate from THF. The GcvT2H2P2 glycine cleavage system produces also N^5,N^{10}-methylene-tetrahydrofolate from THF and glycine.

If SAM is used for other reactions e.g. by AHL synthase or as source of an amino propyl group for the generation of spermidine these reactions yield MTA. MTA is recycled by 5´-methylthioadenosine phosphorylase MtnP and by several other enzymes to methionine. N-butyryl homoserine lactone (BHL) is synthesized by RhlI and N-3-oxododecanoyl homoserine lactone (odDHL) by LasI. Several genes encoding for methionine metabolism enzymes are positively regulated by quorum sensing (indicated by orange plus). Adapted from (Fontecave *et al.*, 2004; Heurlier *et al.*, 2006; Parveen and Cornell, 2011; Sekowska *et al.*, 2004)

3.2 Results & Discussion

3.2.1 Isolation of methionine auxotrophic clinical *Pseudomonas aeruginosa* cystic fibrosis isolates

During routine examinations samples were taken to investigate the frequency of methionine auxotrophic *P. aeruginosa* CF isolates in the respiratory tract of CF patients' (Fig. 18 A). Complex media was used for the selection of *P. aeruginosa* strains. The isolated *P. aeruginosa* strains were further analysed for growth on minimal media. Strains, which did not grow on minimal media were tested for growth on minimal media supplemented with methionine. Methionine auxotrophic strains, which did not grow on minimal media, but grew on minimal media containing methionine were further analysed. Prototrophic strains, which grew on minimal media itself, were not further analysed.

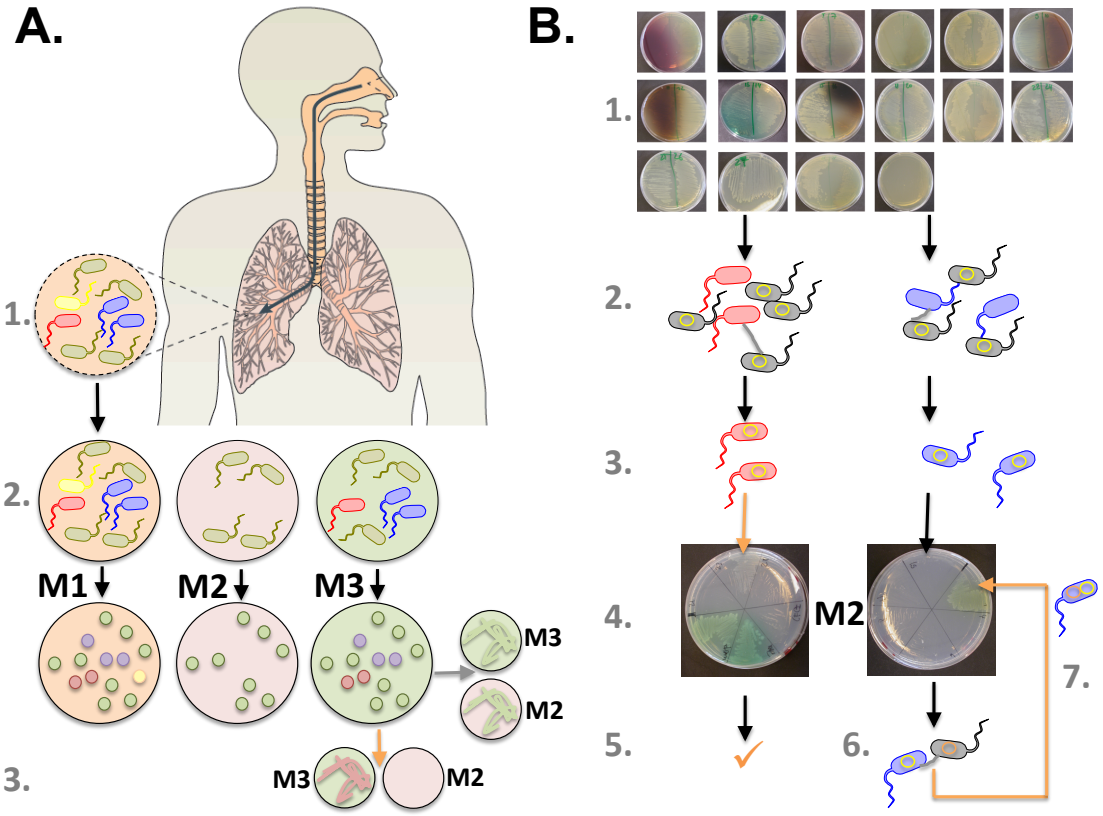


Fig. 18: Isolation of methionine auxotrophic clinical *Pseudomonas aeruginosa* cystic fibrosis isolates and identification of the mutation causing methionine auxotrophy

From patient 1-12 and patient A-D in total 35 clinical *P. aeruginosa* CF strains were isolated, of which 30 were identified as methionine auxotrophic. 14 strains were successfully complemented by pUCP20T*::metF*.

(A.) The strains were isolated from CF patients' during routine examinations (1). Isolated strains were selected on complex medium (M1) and were analysed for growth on minimal medium (M2) and minimal medium containing methionine (M3) (2). Strains, which grew on minimal medium containing methionine (M3) and did not grow on minimal medium (M2), were selected for identification of the cause of methionine auxotrophy (orange arrow) (3). Prototrophic strains, which grew on both minimal medium were not further analysed (grey arrow).

(B.) The methionine auxotrophic strains appeared morphologically very diverse (1). To identify the mutation causing methionine auxotrophy a diparental mating with *E. coli* ST18 carrying the pST10 (pUCP20T*::metF*) was performed (2). Successfully by pST10 plasmid complemented methionine auxotrophic *P. aeruginosa* strains (red and purple) were selected (3) and verified by growth on minimal medium containing carbenicillin (M2) (4). Successfully complemented strains (red) were able to grow on minimal medium (M2) (5). Strains (purple), which were not successfully complemented by pST10 failed to grow on minimal medium (M2) (4). These non-complemented strains containing the pST10 plasmid were complemented by diparental mating with a cosmid library (6). Successful complementation was verified by growth of the respective strains on the minimal medium (M2) containing carbenicillin and tetracycline (orange arrows) (7).

Depiction of human airway model (A) was modified (Folkesson *et al.*, 2012; Kleinstreuer *et al.*, 2008).

35 clinical *P. aeruginosa* strains were isolated in total, of which 30 were identified as methionine auxotrophic. The strains CF-1 to CF-4 (patient B) and KI-AW 13 (patient 6) were not methionine auxotrophic.

3.2.2 Single-nucleotide polymorphisms analysis of clinical *Pseudomonas aeruginosa* cystic fibrosis isolates

The clinical CF strains were tested for clonal variability using binary arrays based on single-nucleotide polymorphism (SNP) genotypes, as described previously (Wiehlmann *et al.*, 2007). The clinical isolates KI-AW 1 to KI-AW 28, CFCZ, CF-1 to CF-4, 65 and BT72 were grouped according to the patients they were isolated from (Table 12). Moreover, clone and clone type were determined, if possible with SNP analysis.

13 different clone types were isolated from 15 different patients. Patient B with the strains CF-1 to 4 and KI-AW 13 of patient 6 were not methionine auxotrophic and were therefore not further analysed. From the remaining 14 patients the clones A, B, E, I, M and P were isolated (Fig. 19 A. & B.). From nine patients (patient 1, 5, 7, 9, 10, 11, 12, A, C) only one *P. aeruginosa* strain was isolated. In contrast, seven methionine auxotrophic *P. aeruginosa* strains were obtained from patient 3.

Table 12: Single-nucleotide polymorphism analysis of clinical *P. aeruginosa* cystic fibrosis isolates

Patient	Colonizing strains	Clone[A]	Clone type[A]
Patient 1	KI-AW 1	P	2C2A
Patient 2	KI-AW 2, KI-AW 3	B	E429
Patient 3	KI-AW 4, KI-AW 5, KI-AW 6, KI-AW 7, KI-AW 8, KI-AW 9, KI-AW 21	P	6C2A
Patient 4	KI-AW 10, KI-AW 11	I	F429
Patient 5	KI-AW 12	I	F429
Patient 6	KI-AW 13°, KI-AW 14, KI-AW 15, KI-AW 26, KI-AW 27	E	1BAE
Patient 7	KI-AW 16		3BAA
Patient 8	KI-AW 17, KI-AW 18, KI-AW 19, KI-AW 23, KI-AW 24		441A
Patient 9	KI-AW 20	M	6E12
Patient 10	KI-AW 22		2D92
Patient 11	KI-AW 25		6862
Patient 12	KI-AW 28		2F82
Patient A	CFCZ		E59A
Patient B	CF-1°, CF-2°, CF-3°, CF-4°	P	6C2A
Patient C	65	A	D421
Patient D	BT72	ND	ND

[A] Clone and clone type according to single-nucleotide polymorphism (SNP) of seven conserved loci and two multiallelic loci (Wiehlmann *et al.*, 2007)

°These strains are not methionine auxotrophic.

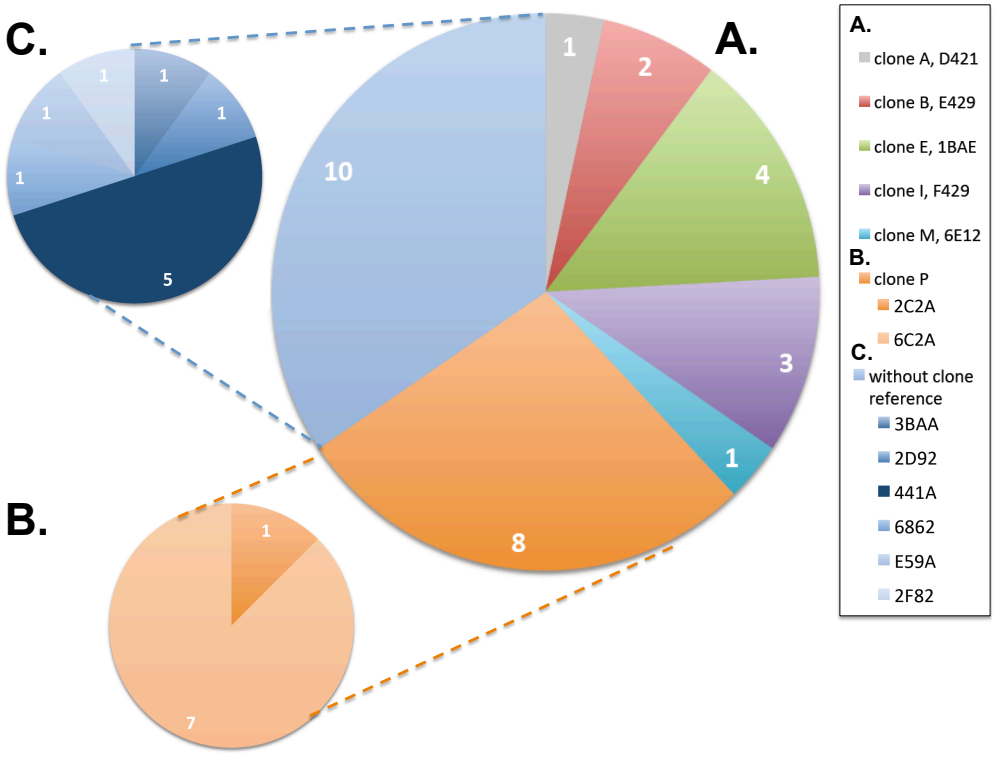


Fig. 19: Distribution of clone and clone type of methionine auxotrophic *P. aeruginosa* cystic fibrosis isolates

30 methionine auxotrophic strains of 14 different patients were characterised by SNP analysis. Six different clones (A) and 13 different clone types (A,B,C) were obtained from patient 1-12, patient A and patient C. From each patient one up to a maximum of seven methionine auxotrophic strains were isolated. Each patient was only colonized by one methionine auxotrophic clone type. The different clones with clone type are shown (A). The distribution of clone types of clone P is indicated (B). Ten strains did not correspond to any previously described clones (reference clone) and were therefore only characterised by clone type (C). The strains CF-1, CF-2, CF-3 and CF-4 of patient B and KI-AW 13 of patient 6 are not depicted here, since the strains are not methionine auxotrophic.

Only one clone and/or clone type was isolated for each patient, respectively. The characterised strains of each patient showed the same SNP pattern in the array. However, the colonies appeared morphologically very different (Fig. 18 B). Notably, KI-AW 13 is a prototrophic strain, whereas the other colonizing strains of patient 6 are methionine auxotrophic. But all strains of patient 6 are classified as clone E and clone type 1BAE. Characterisation of the isolates revealed that ten strains did not correspond to any previously described clones (reference clone) (Wiehlmann *et al.*, 2007). Therefore only the clone type could be determined (Fig. 19 C.). The clone types 441A, 3BAA and 2D92 were not described by their previous study. Moreover, the clone type of 2C2A of clone P was also not represented in their previous study. The most frequent strain in this study belonged to clone P with 8 isolates (Fig. 19 B.). The majority of clone P strains of in total 7 strains belonged to the clone type 6C2A. 6C2A was isolated form CF patients in various places in Germany during the last 17 years (Wiehlmann *et al.*, 2007).

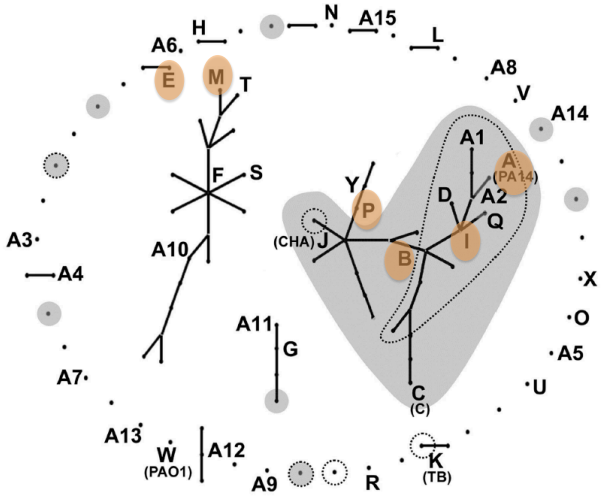


Fig. 20: Clonal complex structures of 240 *P. aeruginosa* strains (adapted from Wiehlmann *et al.* (2007))

240 *P. aeruginosa* strains were collected from various habitats and geographical sources by Wiehlmann *et al.* (2007). The clones high lighted in orange were identified in our study of 30 methionine auxotrophic clinical *P. aeruginosa* CF isolates from CF centres in Germany (Hannover, Munich and Tübingen).

Clones shaded in grey contain *oriC* allele 1 and clones encircled by dots are *exoU*-positive (Wiehlmann *et al.*, 2007).

Most isolates in this study were already present in the MHH strain library. Exceptions are mentioned above. The majority of the MHH strain library was isolated from clinical sources or directly from CF patients in the US and European countries (Wiehlmann *et al.*, 2007). Clone A, which represents the PA14 group was the most prevalent strain in the study by Wiehlmann *et al.* (2007) and also the clone M was very frequent. This study also detected one strain of each clone.

Despite the small sample size of 30 methionine auxotrophic CF isolates and very limited geographical sampling this study is in accordance with previous studies (Fig. 20), which reported that the major clones were detectable in representative samples from both clinical and environmental habitats (Pirnay *et al.*, 2002; Pirnay *et al.*, 2005; Wiehlmann *et al.*, 2007).

3.2.3 Phenotypic characterisation of methionine auxotrophic clinical *Pseudomonas aeruginosa* cystic fibrosis isolates

The mutator frequency among the methionine auxotrophic clinical *P. aeruginosa* CF isolates was investigated. Rifampicin resistance was used as indicator for the mutator phenotype and was determined as colony forming units (CFU) of rifampicin 100 µg/ml (RA-100) resistant bacteria versus total CFU after growth for 24 h. Mutation frequency of clinical *P. aeruginosa* CF isolates showed a strong variation in previous studies (Ciofu *et al.*, 2010; Hall and Henderson-Begg, 2006; Hogardt *et al.*, 2007; Mena *et al.*, 2008; Oliver *et al.*, 2000). Therefore, the terms defined by Ciofu *et al.* (2010) were modified in accordance with the results for PAO1 and PAO1 Δ*mutS* strain. All strains with a mutation frequency ≥ 1.5×10^{-7} were classified as strong mutators. Strains with lower mutation frequency of < 1.5×10^{-7} to ≥ 1.5×10^{-8} were described as weak mutators. A mutation frequency of < 1.5×10^{-8} similar to the *P. aeruginosa* PAO1 was considered as non-mutator. 57 % of the investigated methionine auxotrophic clinical isolates were characterised as strong mutators. 11 % were classified as weak mutators and 32 % as non-mutators (Table 14). *P. aeruginosa* PAO1 Δ*mutS* (Hogardt *et al.*, 2006) was used as a strong mutator phenotype reference strain with a mutation frequency of 5×10^{-6} in this study . The methionine auxotrophic deletion mutant PAO1 Δ*metF* was used as a reference strain for the methionine auxotrophic clinical isolates. The gene *metF* was identified as genetic origin of methionine auxotrophy for the majority of isolates (see 3.2.4).

The production of the *P. aeruginosa* quorum sensing molecules the AHLs was investigated. Only 18 % of the investigated methionine auxotrophic clinical isolates produced an average BHL concentration of > 20 ± 8 µM/OD_{578} (Table 13). PAO1 produced 48.3 ±16.4 µM/OD_{578} BHL (Table 14).

Table 13: Summary of positive phenotypic characteristics according to phenotype

Phenotype	BHL positive*	odDHL positive*	Swimming positive*	Swarming positive*	Twitching positive*
Mutator phenotype (16/28)	19 %	13 %	13 %	25 %	6 %
Weak mutator phenotype (3/28)	0 %	33 %	33 %	33 %	33 %
Non-mutator phenotype (9/28)	22 %	11 %	55 %	33 %	11 %
Total positive strains (phenotype independent)	**18 %**	**14 %**	**29 %**	**25 %**	**11 %**

***** Positive phenotypic characteristics according to definitions in Table 14.

In addition 14 % of the investigated methionine auxotrophic clinical isolates produced an average odDHL concentration of > 10 ± 3 nM/OD_{578}. In contrast, the detected average odDHL concentration for PAO1 was 147.3 ± 3.9 nM/OD_{578}. For PAO1 Δ*metF* an average odDHL concentration of 188.9 ± 26.9 nM/OD_{578} was determined. For 86 % of the investigated methionine auxotrophic clinical isolates nearly any odDHL was detectable (Table 13).

KI-AW 1 (weak mutator) and KI-AW 20 (non-mutator) were the only strains, for which both BHL and odDHL were detected (Table 13).

The motility among the methionine auxotrophic clinical CF strains was investigated. 29 % showed swimming motility comparable to the PAO1. 25 % of the methionine auxotrophic clinical isolates were able to swarm like the PAO1. 11 % of the methionine auxotrophic clinical isolates

were able to twitch like the PAO1. *P. aeruginosa* PAO1 and PAO1 Δ*metF* showed comparable motility. KI-AW 22 was the only methionine auxotrophic clinical CF strain, which was positive for swimming, swarming and twitching.

The majority of the methionine auxotrophic clinical CF strains possessed a weak mutator (11%) to mutator phenotype (57%). No correlation between mutator phenotype and the frequency of reduced production of BHL and odDHL was observed (Table 13). Interestingly, the average BHL concentration was 2- to 3-fold decreased in the majority of the methionine auxotrophic clinical isolates compared to PAO1 wt. In contrast, the average odDHL concentration was 30- to 50-fold decreased in the majority of the methionine auxotrophic clinical isolates compared to PAO1.

This is in accordance with other studies, which described the loss of a functional quorum sensing system, especially of the LasI / LasR system (D'Argenio *et al.*, 2007; Diggle *et al.*, 2007a; Heurlier *et al.*, 2006; Smith *et al.*, 2006; Tingpej *et al.*, 2007).

Ciofu *et al.* (2010) performed a genetic analysis of late phase chronic CF infection isolates and proposed that the mutator phenotype is independent of the selection of *lasR* mutants, since the *lasR* mutants possess a growth advantage due to better nutrient utilization (D'Argenio *et al.*, 2007; Sandoz *et al.*, 2007; Wilder *et al.*, 2011).

We also observed the by Ciofu *et al.* (2010) proposed mutator phenotype independent selection of mucoid phenotype. The exopolysaccharide probably protects the mucoid strains from reactive oxygen species, the host´s immune response and antibiotics (Govan and Nelson, 1993).

The loss of swimming motility was more frequently observed in mutators and weak mutators than among non-mutators (Table 13). For twitching and swarming motility no such effect was observed. In previous studies the abolishment of swimming motility was described to confer resistance to phagocytosis *in vitro* and *in vivo* (Amiel *et al.*, 2010; Balloy *et al.*, 2007). Amiel *et al.* (2010) showed that the loss of flagella function and not the loss of the flagella apparatus itself was the critical factor associated with resistance to nonopsonic phagocytosis. Thereby, explaining the observed advantage of motility loss associated with higher bacterial burden in the lung.

The strong mutator phenotype and weak mutator phenotype were more frequently detected among the methionine auxotrophic clinical CF isolates than the non-mutator phenotype. The *metF* mutation, which was identified as origin of methionine auxotrophy for the majority of strains, itself does not seem to enhance the mutator frequency since PAO1 Δ*metF* does not show an increased mutator frequency compared to PAO1. Therefore, it is proposed that the methionine auxotrophy is also selected independently of the mutator phenotype.

Table 14: Summary of phenotypic characterisation of methionine auxotrophic clinical *P. aeruginosa* CF isolates sorted according to patient and mutator phenotype

Patient	Colonizing strain	Clone[A]	Clone type[A]	Mutator pheno-type[B*]	BHL[C*] [µM / OD_{578}]	odDHL[D*] [nM / OD_{578}]	Swimming[E*] [mm]	Swarming[F*] [mm]	Twitching[G*] [mm]
Patient 1	**KI-AW 1**	P	2C2A	**1.09E-07**	**28.9**	**26.0**	5.3	6.0	3.0
Patient 4	**KI-AW 10**	I	F429	**4.64E-07**	18.3	**77.9**	**14.7**	4.5	2.2
Patient 7	**KI-AW 16**		3BAA	**7.15E-07**	15.3	1.7	3.3	1.8	**8.7**
Patient 8	**KI-AW 23**		441A	**7.23E-07**	23.2	2.2	4.7	5.3	5.3
Patient 9	KI-AW 20	**M**	6E12	2.80E-08	**38.2**	**70.8**	2.2	3.0	3.3
Patient 10	KI-AW 22		2D92	4.33E-08	18.0	3.2	**21.0**	**10.7**	**8.3**
Patient 11	KI-AW 25		6862	2.36E-08	**48.3**	3.2	1.7	2.3	3.3
Patient A	CFCZ		E59A	3.16E-08	21.2	2.2	5.0	2.7	3.0
Patient 3	KI-AW 4	P	6C2A	8.76E-08	13.1	0.9	7.0	5.3	3.0
	KI-AW 5	P	6C2A	2.97E-08	13.1	4.5	**13.7**	**6.0**	3.2
	KI-AW 6	P	6C2A	**8.20E-06**	20.9	**46.6**	3.0	1.8	3.2
	KI-AW 7	P	6C2A	1.54E-08	27.9	6.0	**12.0**	3.3	2.8
	KI-AW 8	P	6C2A	1.67E-08	26.2	0.3	**13.7**	**7.3**	3.3
	KI-AW 9	P	6C2A	**1.45E-07**	7.1	1.1	**11.0**	**8.3**	3.3
	KI-AW 21	P	6C2A	2.33E-08	28.0	7.2	**12.7**	5.3	3.7
Patient 2	**KI-AW 2**	B	E429	**1.31E-06**	5.7	3.3	4.3	1.5	**8.3**
	KI-AW 3	B	E429	**1.57E-06**	16.7	4.4	3.7	0.5	6.7
Patient 4	**KI-AW 11**	I	F429	**2.77E-06**	**32.3**	7.0	**13.0**	**8.3**	1.7
Patient 5	**KI-AW 12**	I	F429	**1.92E-06**	11.2	1.9	1.5	3.3	3.8
Patient 6	**KI-AW 14**	E	1BAE	**2.67E-06**	6.8	3.7	3.7	**6.3**	5.7
	KI-AW 15	E	1BAE	**8.08E-06**	10.3	2.4	3.3	**8.0**	4.7
	KI-AW 26	E	1BAE	**5.09E-06**	18.0	2.3	2.3	4.3	5.3
	KI-AW 27	E	1BAE	**1.52E-06**	12.9	2.4	4.0	5.0	4.7
Patient 8	**KI-AW 17**		441A	**1.67E-05**	6.4	2.5	3.7	5.3	5.7
	KI-AW 18		441A	**3.71E-06**	18.4	1.9	4.0	4.0	4.3
	KI-AW 19		441A	**1.83E-05**	11.6	0.3	2.7	1.8	4.3
	KI-AW 24		441A	**1.77E-06**	20.0	2.1	1.7	3.3	4.3
Patient 12	**KI-AW 28**		2F82	**2.61E-06**	**32.9**	2.2	1.8	2.3	3.7
Patient C	65	**A**	D421	ND	ND	ND	ND	ND	ND
Patient D	BT72	ND	ND	ND	ND	ND	ND	ND	ND
PAO1 Δ*mutS*		ND	ND	**4.94E-06**	ND	ND	ND	ND	ND
PAO1 Δ*metF*		ND	ND	7.16E-08	**45.1**	**188.9**	**24.7**	**19.7**	**6.3**
PAO1		ND	ND	1.54E-08	**48.3**	**147.3**	**25.3**	**19.7**	**8.0**

[A] Clone and clone type according to single-nucleotide polymorphisms (SNPs) at seven conserved loci and two multiallelic loci (Wiehlmann *et al.*, 2007)

[B] Mutator phenotype was determined as CFU of rifampicin 100 µg/ml (RA-100) resistant bacteria vs. CFU. Strains with a mutator frequency ≥ 1.5 x 10^{-7} were classified as strong mutators (**bold black**). Strains with a mutator frequency < 1.5 x 10^{-7} to ≥ 1.5 x 10^{-8} were classified as weak mutators (**bold grey**). Strains with mutator frequency of < 1.5 x 10^{-8} (plain black) were classified as non-mutator. Experiments were performed by Matthias Rottmann (this laboratory, unpublished work).

[C] BHL as µM / OD_{578} was determined. Results above the average BHL concentration of > 20 ± 8 µM / OD_{578} are marked in bold letters.

[D] odDHL as nM / OD_{578} was determined. Results above the average odDHL concentration of > 10 ± 3 nM / OD_{578} are marked in bold letters.

[E] Swimming as the radius [mm] of the respective swimming halo was determined. Results above the average radius of > 6.5 ± 1 mm are marked in bold letters.

[F] Swarming as the radius [mm] of the respective swarming halo was determined. Results above the average radius of > 4.5 ± 1 mm are marked in bold letters.

[G] Twitching as the radius [mm] of the respective twitching halo was determined. Results above the average radius of > 4.5 ± 1 mm are marked in bold letters.

* Results of at least three independent experiments are shown. For the standard deviation of the mean see Appendix Table 31.

ND, not determined

3.2.4 Identification of the mutation causing methionine auxotrophy

In order to identify the mutation causing the methionine auxotrophy, clinical *P. aeruginosa* CF isolates were investigated on minimal media. Auxotrophic strains, which did not grow on M9 20 mM succinate minimal medium (M2), but grew with supplementation of 50 µM methionine (M3) were further analysed (Fig. 18 A).

Strains were also analysed for growth on L-serine, L-homoserine, O-succinyl-L-homoserine, L-cysteine or Hcy supplemented M9 minimal medium, respectively. *P. aeruginosa* PAO1 was able to take up the respective compounds. But none of the investigated methionine biosynthesis precursors was able to support growth on M9 minimal medium. All strains required methionine exclusively for growth. Therefore, the methionine auxotrophy causing defect seemed to be located at the end of the methionine biosynthesis pathway (Fig. 17). Since none of the early precursors was able to complement the methionine auxotrophic strains.

The final step of methionine biosynthesis is the conversion of Hcy via either *metE* or *metH* gene product to methionine. Both enzymes use the cofactor Me-THF and Me-THP as methyl-group donor, respectively.

The mutated gene causing methionine auxotrophy in the strains 65 and BT72 was identified by complementation with a *P. aeruginosa* PAO1 cosmid library (Schobert and Görisch, 1999). A 16.3 kb fragment (genomic position 474648 – 490920) was isolated and sequenced (Thoma, 2009; unpublished). The identified genomic region contained the *metF* (PA0430) gene. PA0430 encodes for a 5,10-methylene tetrahydrofolate reductase (MTHFR). Deletion of *metF* was already described to result in methionine auxotrophy for *E. coli* (Ahmed, 1973). Barth & Pitt (1995) also investigated the high frequency of methionine auxotrophy in strains isolated from the sputum of CF patients. They assumed that the common defect is probably in the conversion of Hcy to Met, but they did not prove it (Barth et al., 1998).

The mutation causing methionine auxotrophy was assumed to be located in the cofactor biosynthesis, most likely in the *metF* gene. MetF converts N^5,N^{10}-methylene-tetrahydrofolate to Me-THF or N^5,N^{10}-methylene-tetrahydropteroyltriglutamate to Me-THP. Both Me-THF and Me-THP are used as cofactors for methionine biosynthesis. To investigate if *metF* can complement methionine auxotrophy in strain 65 and BT72, the plasmid pST10 (pUCP20T*::metF*) was constructed. The complementation of strain 65 and BT72 was verified by growth on M9 minimal medium containing carbenicillin. In addition all methionine auxotrophic strains were complemented with pST10. 14 strains were complemented by *metF* gene (pST10), 2 strains were not complemented by pST10 but were complemented by a plasmid containing the genes *metF* and *metH* (pFH04) and 14 strains were not complemented (Table 15).

The nucleotide sequence and length of the *metF* gene of all methionine auxotrophic isolates was determined and analysed for deviations. The nucleotide sequence length of the wt *metF* gene is 873 bp. The average nucleotide sequence length of the *metF* gene was 868 ± 17 bp in the 30 investigated methionine auxotrophic strains.

The majority of isolates had one up to six mutations in the nucleotide sequence of the *metF* gene. For patient 3 several isolates with the varying frequency of five (KI-AW 5, KI-AW 9, KI-AW 21), six (KI-AW 6, KI-AW 7, KI-AW 8) and twelve (KI-AW 4) mutations in the *metF* gene were

obtained. The frequency of mutator phenotype among the clinical isolates was determined. But no correlation between frequency of mutations and mutator phenotype was observed (Table 15 & Table 16). Interestingly, KI-AW 4 (twelve mutations in the *metF* gene) was not identified as mutator, but KI-AW 6 and KI-AW 9 were identified as strong mutators.

The average nucleotide mutation frequency in the *metF* gene was 3.7 ± 2.4 in the 30 investigated methionine auxotrophic strains. Besides the detection of the deviation in nucleotide sequence, the deviations were further classified either as point mutation, as deletion or as insertion. The average frequency of point mutations in the *metF* gene was 3.1 ± 2.5. So the majority of deviations were point mutations. Eight deletions and seven insertions were detected in total. KI-AW 17 *metF* harbours both an insertion and a deletion. All other strains encountered either deletion or insertion in the *metF* gene, besides point mutations. KI-AW 18, KI-AW 22, KI-AW 23, and KI-AW 24 had only a deletion as mutation. In general the mutations on nucleotide level in the *metF* gene were very diverse and no mutational hotspot was identified. For a detailed analysis see Appendix Table 32.

The outcome of the nucleotide mutations on the amino acid level was analysed (Table 16). Deviations were classified either as amino acid exchange, as frame shift or as stop codon. The amino acid sequence length of the *P. aeruginosa* PAO1 wt MetF protein is 290 aa. A drastic influence of the mutations on the probable average MetF protein length was observed, which was reduced to 147 ± 82 bp in the 30 investigated methionine auxotrophic strains. Most investigated MetF proteins were probably truncated by the stop codon, resulting in shortened proteins.

The shortest protein length identified was 29 aa of strain KI-AW 3, followed by 40 aa for KI-AW 4. The longest truncated protein had an amino acid length of 284 aa determined for KI AW-16, but with 2 amino acid exchanges. This was followed by 178 aa for KI-AW 1. KI-AW 1 was complemented by pST10.

This effect is also reflected by the average total amino acid deviation frequency. Included was the amino acid exchange, frame shift and stop codon insertion in the respective MetF protein. The average total amino acid deviation frequency was 1.9 ± 0.9 incidents per MetF protein in the 30 investigated methionine auxotrophic strains.

Moreover, the strains KI-AW 16 and KI-AW 28 had two and KI-AW 19 had even three amino acid exchanged. 11 of 30 methionine auxotrophic strains had one amino acid exchanged. Frame shift occurred in 16 strains, and was always counted as one event. Due to changed nucleotide sequence, insertions and deletions stop codons were observed in 24 of 30 investigated cases. CFCZ of patient A was the only strain with silent mutations in the nucleotide sequence without any resulting amino acid deviations.

Table 15: Summary of sequence analysis of mutations in the *metF* gene (PA0340) on nucleotide level of clinical methionine auxotrophic *P. aeruginosa* CF isolates

Patient	Colonizing strain	Mutator pheno-type[A]	Length of nucleotide seq. [bp]	Mutations total[B]	Point mutations[C]	Deletion[C]	Insertions[C]	Compl. by pST10/ pFH04[D]
Patient 1	**KI-AW 1**	**++**	873	4	4	0	0	+
Patient 2	**KI-AW 2**	**++**	**874**	**5**	**4**	**0**	**1**	
	KI-AW 3	**++**	**788**	**6**	**5**	**0**	**1**	
Patient 3	KI-AW 4	-	894	12	11	0	1	
	KI-AW 5	-	860	5	5	0	0	
	KI-AW 6	**++**	**860**	**6**	**6**	**0**	**0**	
	KI-AW 7	-	860	6	6	0	0	+
	KI-AW 8	-	860	6	6	0	0	
	KI-AW 9	**++**	860	5	5	0	0	
	KI-AW 21	-	860	5	4	1	0	
Patient 4	**KI-AW 10**	**+**	873	3	3	0	0	+
	KI-AW 11	**++**	**873**	**4**	**4**	**0**	**0**	+
Patient 5	**KI-AW 12**	**++**	**872**	**4**	**3**	**1**	**0**	++
Patient 6	**KI-AW 14**	**++**	**873**	**3**	**3**	**0**	**0**	+
	KI-AW 15	**++**	**873**	**4**	**4**	**0**	**0**	+
	KI-AW 26	**++**	**873**	**2**	**2**	**0**	**0**	+
	KI-AW 27	**++**	**873**	**2**	**2**	**0**	**0**	+
Patient 7	**KI-AW 16**	**+**	874	3	2	0	1	
Patient 8	**KI-AW 17**	**++**	**873**	**2**	**0**	**1**	**1**	+
	KI-AW 18	**++**	**872**	**1**	**0**	**1**	**0**	+
	KI-AW 19	**++**	**872**	**4**	**3**	**1**	**0**	++
	KI-AW 23	**+**	863	1	0	1	0	
	KI-AW 24	**++**	**872**	**1**	**0**	**1**	**0**	+
Patient 9	KI-AW 20	-	874	3	2	0	1	
Patient 10	KI-AW 22	-	863	1	0	1	0	
Patient 11	KI-AW 25	-	873	1	1	0	0	
Patient 12	KI-AW 28	-	874	2	1	0	1	+
Patient A	CFCZ		873	2	2	0	0	
Patient C	65	ND	873	4	4	0	0	+
Patient D	BT72	ND	873	4	4	0	0	+
	PAO1	-	**873**	0	0	0	0	

For detailed analysis see Appendix Table 32.

[A] Mutator phenotype was determined as CFU of rifampicin 100 µg/ml (RA-100) resistant bacteria vs. CFU. Strains with a mutator frequency ≥ 1.5 x 10^{-7} were classified as strong mutators (**++**). Strains with a mutator frequency < 1.5 x 10^{-7} to ≥ 1.5 x 10^{-8} were classified as weak mutators (+). Strains with mutator frequency of < 1.5 x 10^{-8} were classified as non-mutator (-). For detailed information see Table 14.

[B] Mutations total is the sum of all point mutations, deletions and insertions occurring in the *metF* gene of the respective strain.

[C] Point mutation is the exchange of a single nucleotide with another nucleotide. Deletion is the removal of a single nucleotide or several nucleotides. Insertion is the addition of a single nucleotide or several nucleotides.

[D] Clinical isolates were complemented with pST10 (pUCP20T::*metF*) and selected for growth on minimal media. Successful complementation with pST10 is indicated as (+). Strains, which were non-complemented by pST10 but successfully complemented with pFH04 (pUCP20T:: *metF*::*metH*) are indicated as (++).

Table 16: Summary of sequence deviations in the MetF protein (PA0340) on amino acid level of clinical methionine auxotrophic *P. aeruginosa* CF isolates

Patient	Colonizing strain	Mutator pheno-type[A]	Length of amino acid seq. [aa]	Total amino acid deviations[B]	Amino acids exchanged[C]	Frame shift[C]	Stop codon[C]	Compl. by pST10/ pFH04[D]
Patient 1	**KI-AW 1**	**++**	178	1	0	0	1	+
Patient 2	**KI-AW 2**	**++**	**159**	**2**	**0**	**1**	**1**	
	KI-AW 3	**++**	**29**	**2**	**0**	**1**	**1**	
Patient 3	KI-AW 4	-	40	2	0	1	1	
	KI-AW 5	-	164	2	0	1	1	
	KI-AW 6	**++**	**164**	**3**	**1**	**1**	**1**	
	KI-AW 7	-	164	3	1	1	1	+
	KI-AW 8	-	164	3	1	1	1	
	KI-AW 9	**++**	164	2	0	1	1	
	KI-AW 21	-	164	2	0	1	1	
Patient 4	**KI-AW 10**	**+**	290	1	1	0	0	+
	KI-AW 11	**++**	**290**	**1**	**1**	**0**	**0**	+
Patient 5	**KI-AW 12**	**++**	**168**	**3**	**1**	**1**	**1**	++
Patient 6	**KI-AW 14**	**++**	**84**	**1**	**0**	**0**	**1**	+
	KI-AW 15	**++**	**84**	**2**	**0**	**0**	**1**	+
	KI-AW 26	**++**	**84**	**1**	**0**	**0**	**1**	+
	KI-AW 27	**++**	**84**	**1**	**0**	**0**	**1**	+
Patient 7	**KI-AW 16**	**+**	284	2	2	0	1	
Patient 8	**KI-AW 17**	**++**	**61**	**2**	**0**	**1**	**1**	+
	KI-AW 18	**++**	**61**	**2**	**0**	**1**	**1**	+
	KI-AW 19	**++**	**61**	**2**	**0**	**1**	**1**	++
	KI-AW 23	**+**	61	2	0	1	1	
	KI-AW 24	**++**	**61**	**2**	**0**	**1**	**1**	+
Patient 9	KI-AW 20	-	160	2	1	0	1	
Patient 10	KI-AW 22	-	165	2	0	1	1	
Patient 11	KI-AW 25	-	290	1	1	0	0	
Patient 12	KI-AW 28	-	156	4	3	0	1	+
Patient A	CFCZ	-	290	0	0	0	0	
Patient C	65	ND	290	1	1	0	0	+
Patient D	BT72	ND	290	1	1	0	0	+
	PAO1	-	**290**	0	0	0	0	

For detailed analysis see Appendix Table 33

[A] Mutator phenotype was determined as CFU of rifampicin 100 µg/ml (RA-100) resistant bacteria vs. CFU. Strains with a mutator frequency ≥ 1.5 x 10^{-7} were classified as strong mutators (**++**). Strains with a mutator frequency < 1.5 x 10^{-7} to ≥ 1.5 x 10^{-8} were classified as weak mutators (+). Strains with mutator frequency of < 1.5 x 10^{-8} were classified as non-mutator (-). For detailed information see Table 14

[B] The total amino acid deviations are the sum of all amino acid exchanged, frame shifts and stop codon occurring in the MetF protein of the respective strain.

[C] The amino acid exchanged are the replacement of one amino acid by another amino acid due to a change in the triplet codon caused by a nucleotide exchange. The frame shift is a change in the reading frame of the triplet codon caused by an insertion of deletion of a nucleotide, which results in a completely different translation. The stop codon is a triplet in the mRNA, which indicates termination of translation encoded by the codons TAG, TAA and TGA.

[D] Clinical isolates were complemented with pST10 (pUCP20T*::metF*) and selected for growth on minimal media. Successful complementation with pST10 is indicated as (+). Strains, which were non-complemented by pST10 but successfully complemented with pFH04 (pUCP20T*:: metF::metH*) are indicated as (++). For detailed analysis see Appendix Table 32.

KI-AW 10, KI-AW 11, KI-AW 25, 65 and BT72 had only one amino acid exchange in an otherwise not mutated protein. Amino acid 271 tyrosine was exchanged by histidine (Y 271 H) in KI-AW 10 and KI-AW 11 of patient 4. An uncharged amino acid with polar residues (hydroxyl group) was exchanged by a charged amino acid with polar residues (amino group). Amino acid 80 leucine was exchanged by proline (L 80 P) in KI-AW 25. Proline acts as a structural disruptor. In strain 65 amino acid 110 glycine was exchanged by serine (G 110 S). In BT72 valine 203 was exchanged by glutamate (V 203 E).

Interestingly, all of the above mentioned strains except for KI-AW 25 were complemented by the pST10. Four different loci were discovered, which seem to lead to inactivation of the MetF protein at amino acid position 80, 110, 203 and 271 (Appendix Table 33).

The complementation by a functional MetF protein might have not been successful in several methionine auxotrophic clinical isolates, since other studies described the tetrameric structure of the MetF protein (Guenther *et al.*, 1999; Sheppard *et al.*, 1999). The presence of truncated proteins might interfere with the formation of functional MetF protein complexes.

No mutational hot spot was identified for the amino acid deviations.

3.2.5 Virulence factors and quorum sensing in methionine metabolism *Pseudomonas aeruginosa* PAO1 deletion strains

The production of the virulence factor pyocyanin, the iron chelator pyoverdin and the AHL quorum sensing molecules BHL and odDHL was investigated for *P. aeruginosa* strains grown in M9 0.5 % caseinate without further methionine supplementation under microaerobic conditions in stationary phase after 24 h. Stationary phase was used as sampling time point in order to minimize differences caused by slow growing deletion mutants PAO1 Δ*sahH* and PAO1 Δ*mtnP* Δ*sahH* since they reached comparable cell density after 24 h (Supplemental Material Fig. 24). For the growth experiments M9 20 mM succinate with 50 µM methionine supplementation was used.

The methionine auxotrophic deletion mutants PAO1 Δ*metF,* PAO1 Δ*metZ*, PAO1 Δ*metE* Δ*metH* and PAO1 Δ*metE* Δ*metH* Δ*metF* were investigated. Previously, it was discovered that PAO1 Δ*sahH* and PAO1 Δ*mtnP* Δ*sahH* were impaired in growth without methionine supplementation (see 4.2.2). PAO1 Δ*metE*, PAO1 Δ*metH* and PAO1 Δ*mtnP* are not methionine auxotrophic. As control strain for pyocyanin production PA14 Δ*phz* was used, in which both phenazine operons *phzABCDEFG* were deleted.

The pyocyanin production was decreased in all methionine auxotrophic deletion strains (Fig. 21 A). PAO1 Δ*metF* produced 50 % of the PAO1. For PAO1 Δ*metZ* no pyocyanin was detectable. The double deletion mutant PAO1 Δ*metE* Δ*metH* and the triple deletion mutant PAO1 Δ*metE* Δ*metH* Δ*metF* showed decreased pyocyanin production of 20 % and 10 %, respectively. Interestingly, PAO1 Δ*metE* and PAO1 Δ*metH* and PAO1 Δ*mtnP* produced wt like and even increased amounts of pyocyanin. For PAO1 Δ*sahH*, PAO1 Δ*mtnP* Δ*sahH* and the control strain PA14 Δ*phz* no pyocyanin was detectable.

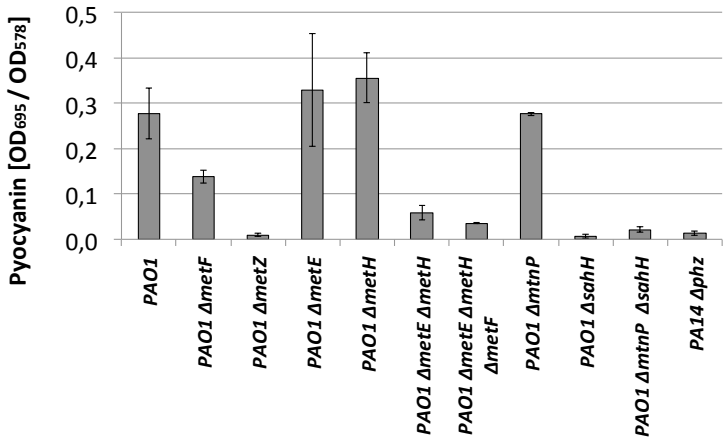
Wild type like pyoverdin production of 2086 ± 142 F_{460}/ OD_{578} was observed for PAO1 Δ*metF* and PAO1 Δ*metZ* (Fig. 21 B). PAO1 Δ*sahH* and PAO1 Δ*mtnP* Δ*sahH* produced only 20 % of the wt. All other strains produced 50 - 60 % of the PAO1 pyoverdin amount.

The production of AHL quorum sensing molecules BHL and odDHL was determined (Fig. 21 C &D). The PAO1 produced 41 ± 6 µM/ OD_{578} BHL. PAO1 Δ*metE*, PAO1 Δ*metH*, PAO1 Δ*metE* Δ*metH* and PAO1 *metE* Δ*metH* Δ*metF* produced wt like amounts of BHL. Interestingly, the double and triple mutant produced slightly increased amounts. PAO1 Δ*metF* and PAO1 Δ*metZ* produced approximately 170 % of the wt with 72 ± 5 µM/ OD_{578} and 68 ± 10 µM/ OD_{578}, respectively. PAO1 Δ*mtnP* also produced increased amounts of BHL of 60 ± 4 µM/ OD_{578}. PAO1 Δ*sahH* and PAO1 Δ*mtnP* Δ*sahH* produced only 44 % and 20 % of the wt BHL amount, respectively.

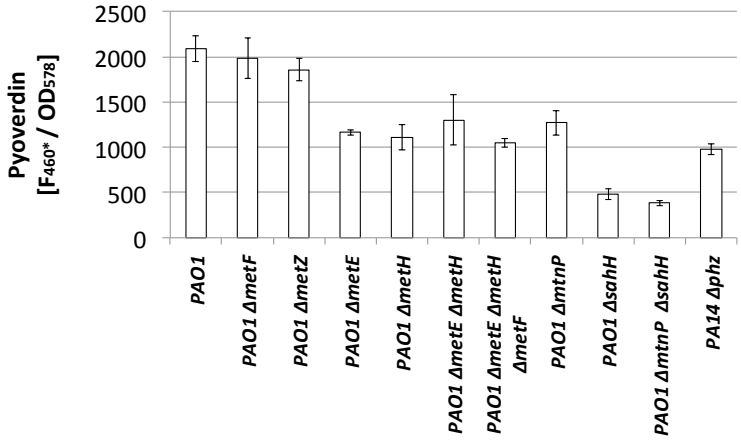
The PAO1 produced 962 ± 89 nM/ OD_{578} odDHL. Unexpectedly, the PAO1 Δ*metF* produced increased amounts of odDHL of 1297 ± 116 nM/ OD_{578}. In contrast, all other deletion mutants produced only 30 – 40 % of the odDHL normally produced by the PAO1.

The reduced pyocyanin production in the methionine auxotrophic deletion strains was probably due to decreased cellular methionine level or decreased recycling of methionine. This most likely results in an imbalance of the intracellular SAM / SAH level.

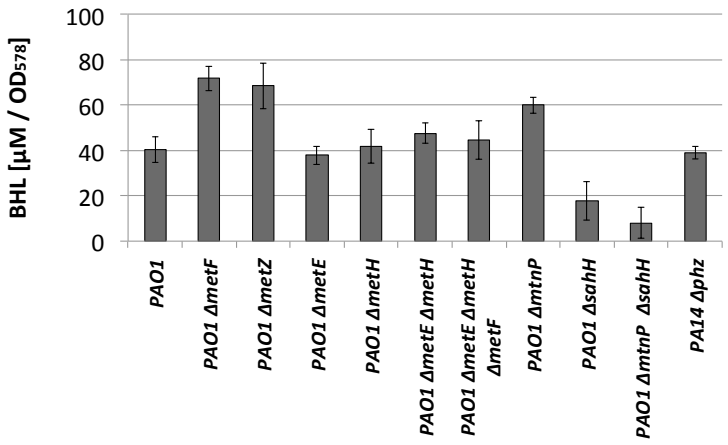
A.



B.



C.



D.

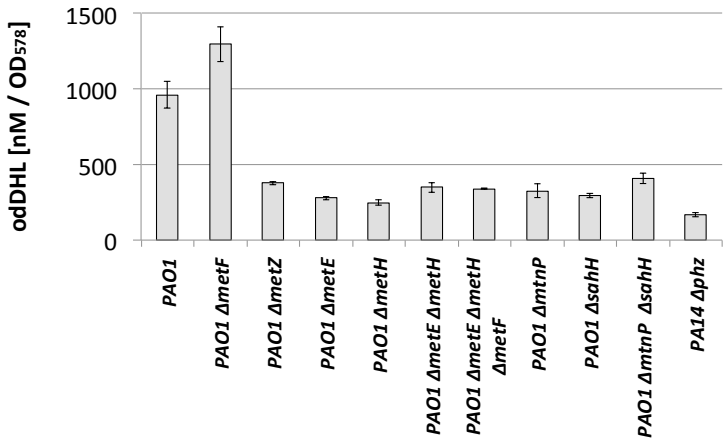


Fig. 21: Production of virulence factors and quorum sensing molecules by the methionine metabolism *P. aeruginosa* deletion strains

In PAO1, PAO1 Δ*metF*, PAO1 Δ*metZ*, PAO1 Δ*metE*, PAO1 Δ*metE* Δ*metH*, PAO1 Δ*metE* Δ*metH* Δ*metF,* PAO1 Δ*mtnP*, PAO1 Δ*sahH and* PA14 Δ*phz* the production of the virulence factors pyocyanin [OD_{695}/ OD_{578}] (A) and pyoverdine [F_{460}*/ OD_{578}] (B) was investigated. We analysed the production of the quorum sensing molecules BHL [µM/ OD_{578}] (C) and odDHL [nM/ OD_{578}] (D). Strains were grown in M9 0.5 % caseinate without further methionine supplementation under microaerobic conditions. Virulence factors were analysed after 24 h of growth.

The standard deviation of three representative experiments is shown.

*F_{460} (Emission of relative fluorescence at 460 nm; Excitement at 405 nm)

Pyocyanin synthesis itself is SAM dependent. Phenazine-1-carboxylic acid is synthesized from chorismic acid via the two *phzABCDEFG* operons (Parsons *et al.*, 2007) (Fig. 22). Phenazine-1-carboxylic acid is methylated by PhzM using SAM to 5-methylphenazine-1-carboxylic acid betaine. 5-Methylphenazine-1-carboxylic acid betaine is further converted by the probable FAD-dependent monooxygenase PhzS in the presence of oxygen and NADH to pyocyanin (Parsons *et al.*, 2007).

The methylation step catalysed by PhzM might be impaired in methionine auxotrophic strains, due to reduced SAM availability. Another possibility might be a low affinity of PhzM towards SAM, which might result in reduced synthesis at low SAM concentrations. Other more essential

cellular reactions might be still catalysed by the respective enzymes, due to the enzymes higher affinity towards SAM. Furthermore, high intracellular SAH concentrations might lead to inhibition of the methylation reactions. Quorum sensing is regulating pyocyanin formation (Gallagher *et al.*, 2002; Lau *et al.*, 2004).

In PAO1 Δ*metF* pyocyanin formation was still possible, but reduced. Even more decreased was the pyocyanin formation in PAO1 Δ*metE* Δ*metH* and PAO1 Δ*metE* Δ*metH* Δ*metF* probably due to the changed intracellular SAM / SAH level.

In contrast, pyocyanin formation was not detected for PAO1 Δ*metZ,* PAO1 Δ*sahH* and PAO1 Δ*mtnP* Δ*sahH*. The assumption is that the SAM / SAH level might prevent the formation in these strains. The quorum sensing molecules BHL and odDHL levels do not seem to be the regulating factor, since other strains with similar odDHL and BHL levels produced pyocyanin. This was e.g. the case for PAO1 Δ*mtnP,* which produced pyocyanin, whereas PAO1 Δ*metZ* did not. The methionine auxotrophic strains are dependent on external methionine, to refill their methionine pool and thereby refilling the SAM pool to be able to grow.

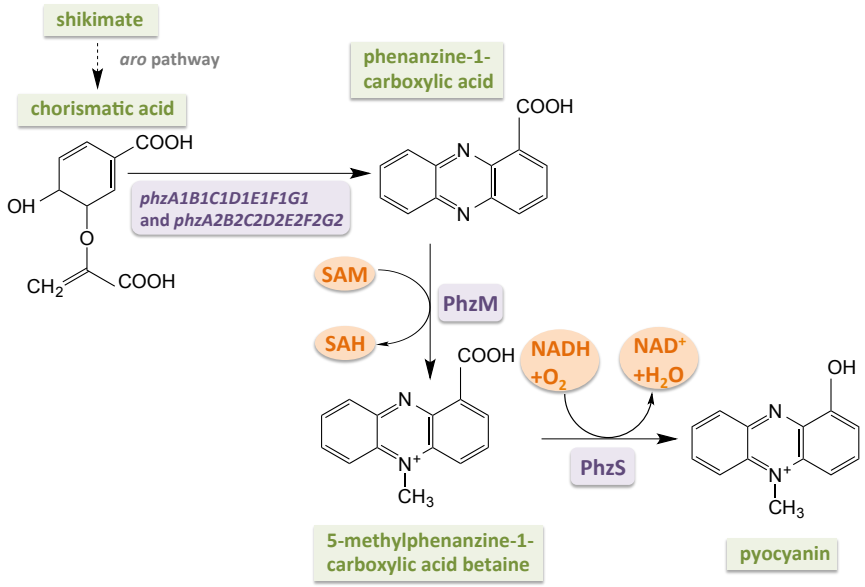


Fig. 22: Pyocyanin synthesis (adapted from Lau *et al.*, 2004)

Pyocyanin is synthesized from chorismatic acid by the *phzABCDEFG* operons. Phenazine-1-carboxylic acid is methylated by PhzM using SAM to 5-methylphenazine-1-carboxylic acid betaine. 5-methylphenazine-1- carboxylic acid betaine is further converted by the probable FAD-dependent monooxygenase PhzS in the presence of oxygen and NADH to pyocyanin (Parsons *et al.*, 2007).

Pyoverdin is a powerful iron Fe^{3+} scavenger and transporter (Schalk and Guillon, 2012). It consists of a peptide moiety of diverse structure, and a conserved fluorescent chromophore, which can carry a variable acyl chain (Meyer, 2000). Pyoverdin synthesis is controlled by the ECF sigma factor PvdS (Vasil, 2007). Pyoverdin synthesis is neither influenced by the deletion of *metF* nor *metZ*, whereas all other deletions reduced its synthesis. All experiments were conducted under iron limiting conditions. During growth in the CF lung with cooperative strains producing the public good pyoverdin these strains would not be influenced in iron availability. PAO1 Δ*metF* and PAO1 Δ*metZ* did not act as a cheater per se. With methionine

supplementation as in M9 0.5% caseinate pyoverdin synthesis was not influenced. But during growth in a methionine depleted environment as during the chronic CF infection this might change.

The odDHL production was only increased in PAO1 Δ*metF*, whereas BHL production was also increased in PAO1 Δ*metF,* PAO1 Δ*metZ* and PAO1 Δ*mtnP.* Due to the deletion PAO1 Δ*metF* probably accumulates Hcy and methylene-THF, PAO1 Δ*metZ* probably accumulates O-succinyl-homoserine and PAO1 Δ*mtnP* cannot recycle MTA.

We propose that these strains might use the synthesis of quorum sensing molecules as salvage pathway to dispose not recyclable Met and SAM reaction residues or to balance the intracellular SAM and methionine level.

Interestingly, odDHL production of the PAO1 wt was 7-fold increased during growth in M9 0.5% caseinate compared to MHB (Table 14), while BHL production remained unchanged. Palmer *et al.* (2007) showed the positive influence of aromatic amino acids on the PQS biosynthesis, previously. The growth medium M9 0.5% caseinate is very rich in amino acids and also in aromatic amino acids. AHL biosynthesis is assumed to take place via fatty acid biosynthesis not via β-oxidation pathway (Dickschat, 2010). The most frequently used starter unit in fatty acid biosynthesis is acetyl-CoA. Dickschat (2010) proposed that also amino acids might serve as starter units for methyl branched AHLs, similar to methyl branched fatty acids from the amino acid-derived starter units. The signaling molecule odDHL is not methyl branched, but biosynthesis is not completely elucidated. Therefore, it can be discussed if odDHL expression is influenced by nutrient availability. But further studies have to be performed to verify this assumption.

3.2.6 Transcriptome analysis of *Pseudomonas aeruginosa* PAO1 and PAO1 Δ*metF* in M9 0.5 % caseinate under microaerobic to anaerobic conditions

The transcriptional profiles of PAO1 were compared to PAO1 Δ*metF* grown in M9 0.5% caseinate under microaerobic to anaerobic conditions. M9 0.5% caseinate was used to simulate the high amino acid content of the sputum of CF patients. The transcriptional profiles were analysed after 4 h and after 12 h of growth. Previous experiments have shown a reduced pyocyanin production by PAO1 Δ*metF* (3.2.5).

The optical density OD_{578} was measured and the colony forming units (CFU) were determined for PAO1 and PAO1 Δ*metF* during growth in M9 0.5% caseinate (Fig. 23 A). Moreover, the protein concentration [mg/ml] and the DNA concentration [µg/ml] were determined (Fig. 23 B). No significant differences in growth behaviour, protein or DNA concentration between PAO1 and PAO1 Δ*metF* were detected. Defined growth conditions were chosen, which did not result in a detectable growth phenotype of PAO1 Δ*metF.* The casein protein was used as source of methionine and supported wt-like growth of PAO1 Δ*metF* without additional methionine supplementation. Therefore, the experiment focused on the transcriptional differences induced by the *metF* deletion. Moreover, the effect of reduced pyocyanin production by PAO1 Δ*metF* was investigated in this model system.

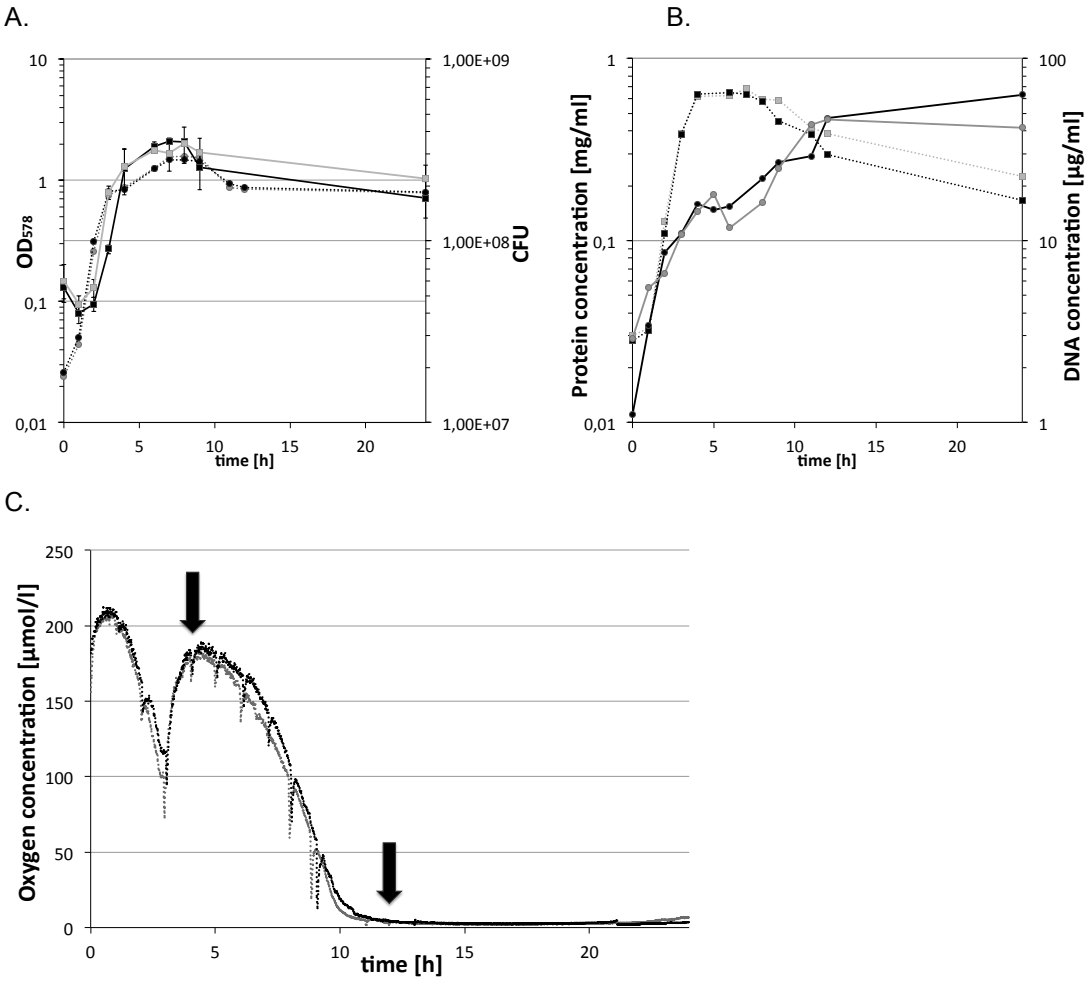


Fig. 23: Transcriptome analysis for PAO1 wt and PAO1 Δ*metF* in M9 0.5 % caseinate under microaerobic to anaerobic conditions

The OD_{578} (circles, dashed line) was determined and the CFU (squares, solid line) for PAO1 (black) and PAO1 Δ*metF* (grey) were determined (A). Moreover, the protein concentration [mg/ml] (squares, dashed line) and DNA concentration [µg/ml] (circles, solid line) was determined for PAO1 (black) and PAO1 Δ*metF* (grey) (B).

The oxygen concentration [µmol/l] was measured during growth of PAO1 wt (black dotted line) and PAO1 Δ*metF* (grey dotted line) in M9 0.5 % caseinate (C). Sampling points for the transcriptome analysis during late exponential phase (4 h) and stationary phase (12 h) are indicated by black arrows. Representative experiments are shown.

The OD_{578} and the CFU showed rapid increase until 3 h (Fig. 23 A). Afterwards OD_{578} increased only 1.7-fold between 3 h and 6 h and remained constant until oxygen depletion at 12 h. Similarly the CFU remained constant between 4 h and 24 h for both PAO1 and PAO1 Δ*metF*.

The oxygen consumption of PAO1 and PAO1 Δ*metF* was evaluated (Fig. 23 C). The conditions ranged form microaerobic after 4 h of growth to anaerobic conditions after 12 h. No significant differences in oxygen consumption between PAO1 and PAO1 Δ*metF* were observed. The PAO1 Δ*metF* culture contained a little less oxygen between 4 h and 12 h than the PAO1 wt. Both strains showed a strong local decrease in oxygen concentration after 3 h. Oxygen was consumed slowly after 4 h. Oxygen was depleted to < 5 µmol after 12 h.

The protein concentration [mg/ml] increased 1.6-fold between 3 h and 4 h (Fig. 23 B). After 4 h the protein concentration remained stable and the DNA concentration increased only very slowly.

The local minimum in oxygen concentration after 3 h might be an indicator of the strong metabolic activity of both strains and the high growth rates. Moreover, the switch from one nutrient source to another due to the depletion of the utilized nutrient source might also be indicated by the local minimum of oxygen concentration followed by the repletion of the oxygen concentration. In a previous study it was observed that different amino acids were taken up und were utilized at different time points from amino acid medium (AAM) (Chapter 2.2.13).

Although medium composition and direct amino acid availability are different between AAM and M9 0.5 % caseinate, switch from one nutrient source to another combined with the end of exponential phase and the entry of stationary phase could be indicated by decrease in oxygen concentration after 3 h. Between 4 h to 12 h bacteria were consuming oxygen, but less rapidly. The protein concentration remained stable. DNA concentration remained also stable between 4 h and 6 h, but increased 3-fold between 6 h and 12 h for both strains, while the CFU remained constant. Several genomes per bacteria are described as an indicator of stationary phase (Akerlund *et al.*, 1995). Therefore, one can assume that both cultures were in late exponential phase or rather at the entry to stationary phase at 4h and in stationary phase after 12 h.

Comparison of the two strains and two time points identified that 1389 genes were differently expressed (Appendix Table 34). 213 genes were upregulated and 82 genes downregulated for PAO1 4 h vs. PAO1 Δ*metF* 4 h (W4 / M4). The comparison of PAO1 12 h vs. PAO1 Δ*metF* 12 h (W12 / M12) identified 137 genes as upregulated and 126 as downregulated in total. 356 genes were upregulated in PAO1 Δ*metF* 12 h vs. PAO1 Δ*metF* 4 h (M12 / M4). 351 genes were downregulated (M12 / M4). 469 genes were upregulated and 571 genes were downregulated in PAO1 12 h vs. PAO1 wt 4 h (W12 / W4).

Energy generation and stress response

During microaerobic to anaerobic growth conditions without supplementation of nitrate or nitrite, arginine normally serves as energy source for growth. Under anaerobic to microaerobic conditions, *P. aeruginosa* can degrade arginine to ornithine via the arginine deaminase (ADI) pathway, which is linked to the generation of ATP by substrate-level phosphorylation (Lu *et al.*, 2004; Mercenier *et al.*, 1980; Vander Wauven *et al.*, 1984). Our complex medium contained other amino acids to serve as carbon sources to allow growth since ornithine is excreted (Vander Wauven *et al.*, 1984).

Interestingly, the *arcDABC* operon was found induced in the PAO1 after 4 h, but was downregulated in PAO1 after 12 h (Table 17). The same expression pattern was observed for the ArgR regulator in PAO1. In contrast the *arcDABC* operon was induced in PAO1 Δ*metF* after 12 h. A similar expression pattern was detected for the arginine succinyltransferase (AST) pathway genes. With the exception that no regulation of the AST pathway genes was observed for PAO1 Δ*metF* (M12 / M4). The AST pathway converts arginine and ornithine to glutamate under aerobic conditions (Itoh, 1997; Park *et al.*, 1997).

Table 17: Microarray analysis of selected genes in PAO1 compared to PAO1 Δ*metF* in M9 0.5 % caseinate

Differentially regulated genes of *P. aeruginosa* PAO1 compared to PAO1 Δ*metF* after 4 h and after 12 h in M9 0.5 % caseinate under microaerobic to anaerobic conditions. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated of PAO1 wt 4 h vs. PAO1 Δ*metF* 4 h (W4 / M4), PAO1 wt 12 h vs. PAO1 Δ*metF* wt 12 h (W12 / M12), PAO1 Δ*metF* 12 h vs PAO1 Δ*metF* 4 h (M12 / M4) and PAO1 wt 12 h vs PAO1 wt 4 h (W12 / W4).

Gene ID[A]	Gene name[A]	Function[A]	FC W4 / M4	FC W12 / M12	FC M12 / M4	FC W12 / W4
Adaptation and Protection:						
PA0059	*osmC*	osmotically inducible protein OsmC	2,3			-2,4
PA0382	*micA*	DNA mismatch repair protein MicA	-2,2			2,2
PA3309	*uspK*	Usp-type stress protein UspK		-4,9	12,1	2,2
PA2532	*tpx*	thiol peroxidase		2,3	-2,3	
Energy metabolism & denitrification:						
PA0105	*coxB*	cytochrome c oxidase, subunit II (aa_3-type)	2,5		-3,4	-4,9
PA0106	*coxA*	cytochrome c oxidase, subunit I (aa_3-type)	3,3	3,5	-7,2	-6,9
PA0107		conserved hypothetical protein	3,6	3,7	-8,6	-8,3
PA0108	*coIII*	cytochrome c oxidase, subunit III	3,1	3,5	-6,4	-5,7
PA0110		hypothetical protein	2,4	2,2	-3,2	-3,4
PA0111		hypothetical protein	2,9	3,2	-5,9	-5,3
PA0112		hypothetical protein	3,0	2,4	-4,7	-6,1
PA0113		probable cytochrome c oxidase assembly factor	3,5	2,7	-5,2	-6,7
PA0114	*senC*	SenC	3,2		-3,5	-6,7
PA0509	*nirN*	probable c-type cytochrome		-2,3	2,4	
PA0510	*nirE*	probable uroporphyrin-III c-methyltransferase		-2,4	2,4	
PA0515	*nirD*	probable transcriptional regulator		-3,0	3,6	
PA1172	*napC*	cytochrome c-type protein NapC	2,9			-5,7
PA1173	*napB*	cytochrome c-type protein NapB precursor	3,4			-6,8
PA1174	*napA*	periplasmic nitrate reductase protein NapA	3,0		-2,1	-6,8
PA1175	*napD*	NapD protein of periplasmic nitrate reductase	3,1		-2,0	-6,9
PA1176	*napF*	ferredoxin protein NapF	2,3		-2,2	-6,8
PA1177	*napE*	periplasmic nitrate reductase protein NapE		-2,0		-2,6
PA1546	*hemN*	oxygen-independent coproporphyrinogen III oxidase		-2,8	6,5	2,5
PA1555		probable cytochrome c		-8,3	16,3	2,3
PA1556		probable cytochrome c oxidase subunit		-6,7	13,9	2,3
PA1557		probable cytochrome oxidase subunit (cbb_3-type)		-6,4	12,8	2,3
PA3930	*cioA*	cyanide insensitive terminal oxidase				-2,9
PA4494	*roxS*	probable two-component sensor		-2,3		
Arginine fermentation:						
PA0893	*argR*	transcriptional regulator ArgR				-2,3
PA5170	*arcD*	arginine/ornithine antiporter	2,7	-2,0	2,6	-2,1
PA5171	*arcA*	arginine deiminase	3,3	-2,6	2,8	-3,1
PA5172	*arcB*	ornithine carbamoyltransferase, catabolic	3,4	-2,6	2,7	-3,1
PA5173	*arcC*	carbamate kinase	3,2	-2,5	3,8	-2,1
Metabolism:						
PA0897	*aruG*	arginine/ornithine succinyltransferase AII subunit	2,1	-2,1		-3,4
PA0898	*aruD*	succinylglutamate 5-semialdehyde dehydrogenase	2,0	-2,0		-3,4
PA0899	*aruB*	succinylarginine dihydrolase	2,1	-2,2		-3,9
PA0901	*aruE*	succinylglutamate desuccinylase		-2,1		-2,6
PA2113	*opdO*	pyroglutamate porin OpdO	2,4		-6,6	-17,9
PA2114		probable major facilitator superfamily (MFS) transporter	2,2		-10,1	-31,2
PA4501	*opdD*	Glycine-glutamate dipeptide porin OpdD		3,6		
PA0887	*acsA*	acetyl-coenzyme A synthetase		-5,4	3,5	
PA2862	*lipA*	lactonizing lipase precursor		4,4	-50,9	-20,0
PA3366	*amiE*	aliphatic amidase	2,2		-11,6	-21,2
PA4813	*lipC*	lipase LipC	2,2		-3,3	-7,0
PA5427	*adhA*	alcohol dehydrogenase		-3,0	4,9	
PQS biosynthesis and anthranilate metabolism:						
PA0997	*pqsB*	Homologous to beta-keto-acyl-acyl-carrier protein syn.*		2,3		
PA0998	*pqsC*	Homologous to beta-keto-acyl-acyl-carrier protein syn.*		2,1		
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III		2,0		
PA2579	*kynA*	2,3-oxidoreductase (decyclizing) KynA		2,5		
PA2080	*kynU*	kynureninase KynU		2,1	-2,7	
PA2081	*kynB*	kynurenine formamidase, KynB		2,5	-2,3	
PA2511	*antR*	probable transcriptional regulator		4,8		3,8
PA2512	*antA*	anthranilate dioxygenase large subunit		3,4		3,7
PA2513	*antB*	anthranilate dioxygenase small subunit		5,3		5,9
PA2514	*antC*	anthranilate dioxygenase reductase		4,2		5,1

Continued:

Gene ID[A]	Gene name[A]	Function[A]	FC W4 / M4	FC W12 / M12	FC M12 / M4	FC W12 / W4
Phenazine biosynthesis:						
PA1901	*phzC2*	phenazine biosynthesis protein PhzC			5,0	7,2
PA1902	*phzD2*	phenazine biosynthesis protein PhzD			6,4	5,9
PA1903	*phzE2*	phenazine biosynthesis protein PhzE			14,2	14,3
PA1904	*phzF2*	probable phenazine biosynthesis protein			18,0	16,5
PA1905	*phzG2*	probable pyridoxamine 5'-phosphate oxidase			21,8	20,0
PA4209	*phzM*	probable phenazine-specific methyltransferase			11,0	13,4
PA4210	*phzA1*	probable phenazine biosynthesis protein			28,4	31,4
PA4211	*phzB1*	probable phenazine biosynthesis protein			13,8	23,3
PA4217	*phzS*	flavin-containing monooxygenase			22,7	28,0
Hydrogen cyanide biosynthesis:						
PA2193	*hcnA*	hydrogen cyanide synthase HcnA			14,9	21,4
PA2194	*hcnB*	hydrogen cyanide synthase HcnB			14,0	10,3
PA2195	*hcnC*	hydrogen cyanide synthase HcnC			9,9	12,0
PA3049	*rmf*	ribosome modulation factor	3,4		3,5	
PA1898	*qscR*	quorum-sensing control repressor		3,0		

[A] Gene ID, gene name, function and PseudoCAP Function Class (PCFC) are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

*Abbreviations: syn. (synthase)

The expression pattern indicates that the PAO1 utilizes arginine very efficiently via the ADI and AST pathway genes after 4 h, whereas arginine must have still been present in the PAO1 Δ*metF* culture after 12 h and was probably still utilized at this time point. This indicates a different anaerobic consumption of arginine by PAO1 and by PAO1 Δ*metF*.

Both genes for the aerobic and microaerobic to anaerobic utilization of arginine were detected with the genes of the AST and ADI pathway expressed for both strains. The ArgR regulator and the presence of exogenous arginine regulate both pathways (Itoh, 1997; Park *et al.*, 1997). Moreover, the *arcDABC* operon is induced by low oxygen tension and Anr the global regulator of the anaerobic gene expression (Gamper *et al.*, 1991; Mercenier *et al.*, 1980). Anr possesses an oxygen sensitive iron-sulphur cluster and directly induces genes required for arginine fermentation and cyanide biosynthesis (Zimmermann *et al.*, 1991). Therefore, the oxygen concentration and the culture conditions were able to induce both pathways. Due to the absence of nitrate / nitrite the *arc* genes were not repressed by the nitrate response regulator NarXL (Benkert *et al.*, 2008).

The change of oxygen concentration during growth was also detected by the change of expression of cytochrome oxidases in both strains. The *cox* operon consisting of the genes *coxBA*, PA0107 and *colII* was downregulated after 12 h for both PAO1 and for PAO1 Δ*metF*, since the oxygen concentration in the cultures was very low (Fig. 23 C), this is in accordance with a previous study (Kawakami *et al.*, 2010). The *cox* operon is described to encode for an aa_3-type oxidase of low oxygen affinity and was shown to be induced by RpoS and repressed by RoxSR (Kawakami *et al.*, 2010; Schuster *et al.*, 2004).

RoxSR is proposed to be regulated directly or indirectly by the redox status of the respiratory chain (Arai, 2011). Moreover, the *cox* operon is described to be regulated by starvation of carbon, nitrogen or iron (Arai, 2011; Kawakami *et al.*, 2010). Interestingly, the expression of the *cox* operon was increased in the PAO1 after 4 h and 12 h compared to PAO1 Δ*metF.* This

indicates that either the oxygen concentration, the oxygen sensing or the underlying regulation pattern must have been either slightly increased or altered in PAO1 vs. PAO1 Δ*metF* culture at both time points. No significant change was visible in the measured oxygen concentration (Fig. 23 C). It could be discussed if this is due to increased expression of the *roxS* gene in PAO1 Δ*metF* compared to PAO1 after 12 h. Besides, the induction of the regulator RpoS in PAO1 Δ*metF* could be altered.

The cbb_3-type 2 oxidase operon (PA1555-PA1557) was upregulated for both PAO1 and PAO1 Δ*metF* after 12 h. After 12 h the induction in PAO1 Δ*metF* was much stronger than in PAO1, since the expression was downregulated for PAO1. The cbb_3-type 2 oxidase is predicted to have a high affinity for oxygen and is expressed at low oxygen levels (Alvarez-Ortega and Harwood, 2007; Kawakami *et al.*, 2010). The transcriptional profile suggested that PAO1 Δ*metF* was using the cbb_3-type 2 oxidase operon preferably after 12 h. At this time point the oxygen concentrations inside each culture was depleted (Fig. 23 C). The cbb_3-type 2 oxidase operon is positively regulated by Anr and RoxSR. This is in accordance with the previous observations.

The gene *cioA* encoding for the cyanide insensitive *bd*-type oxidase (PA3930) was downregulated in PAO1 after 12 h and no change in expression was observed for PAO1 Δ*metF*. The *cio* oxidase operon is only induced if no other terminal oxidase of the heme-copper superfamily is able to function (Arai, 2011). The *cio* oxidase operon is positively regulated by RoxSR and activated to a small degree by RpoS but negatively regulated by Anr (Comolli and Donohue, 2004; Cooper *et al.*, 2003; Kawakami *et al.*, 2010). Interestingly, the cyanide biosynthesis operon *hcnABC* was upregulated in both PAO1 and PAO1 Δ*metF* after 12 h. In agreement, the cyanide biosynthesis is also regulated by Anr and is dependent on microaerobic conditions and the entry of stationary phase (Pessi and Haas, 2000; Williams *et al.*, 2007). It was shown that the amino acids threonine and glycine stimulate cyanide production. Furthermore, cyanide biosynthesis is regulated cell density dependent by the RhlI/R quorum system and GacS/GacA (Reimmann *et al.*, 1997). The question remains why there is no change of expression of *cioA* observed for PAO1 Δ*metF* as the induction of the cyanide biosynthesis is observed. The *cioA* expression might be influenced by an altered regulation of RhlI/R quorum system and GacS/GacA by the increased expression of quorum sensing molecules. The increased production was observed for both BHL and odDHL in PAO1 Δ*metF* after 24 h (3.2.5).

A very strong induction of cbb_3-type 2 oxidase operon in PAO1 Δ*metF* was observed after 12 h. This might be the solution for energy generation by PAO1 Δ*metF* to compensate for the unchanged *cioA* expression and might be stimulated by RoxS. No decrease in CFU was observed for neither PAO1 Δ*metF* nor PAO1. Therefore, the PAO1 Δ*metF* culture seems not to be inhibited by cyanide, if produced. But the actual cyanide production of PAO1 Δ*metF* cultures has to be determined.

Interestingly, the genes *nirE* and *hemN* encoding for the proteins NirE and HemN, which are both SAM dependent enzymes, were upregulated in PAO1 Δ*metF* after 12 h. NirE is a SAM-dependent uroporphyrinogen III methyltransferase, which catalyses the conversion of uroporphyrinogen III to precorrin-2 (Storbeck *et al.*, 2009). HemN catalyses the essential conversion of coproporphyrinogen III to protoporphyrinogen IX during heme biosynthesis (Layer

et al., 2003). HemN is an iron-sulphur cluster containing protein belonging to the radical SAM protein family (Layer *et al.*, 2003; Sofia *et al.*, 2001). The promoter of *hemN* is recognized by both Anr and Dnr (Schreiber *et al.*, 2007).

Besides *nirE*, the genes *nirD and nirN* were also upregulated in PAO1 Δ*metF* after 12 h. NirD is a predicted transcriptional regulator, which also possesses enzymatic properties (Nicke, 2013; Walter, 2011). NirN probably catalyses together with NirF the last step of heme d_1-biosynthesis and cofactor-insertion into NirS (Nicke, 2013). Normally, the *nir* operon is induced by the NO-sensing regulator Dnr. The expression of *nirS* is moreover NarXL regulated. The observed transcriptional upregulation of the genes *nirE* and *hemN* seems to be specific for PAO1 Δ*metF*. One can discuss if the induction is due to either a higher demand for their reaction products or due to reduced availability of both proteins as functional enzymes with SAM as cofactor due to limitation of SAM caused by the unbalanced intracellular methionine level in PAO1 Δ*metF*. But in order to fully understand the underlying regulation it has to be further investigated.

The universal stress protein gene *uspK* and the alcohol dehydrogenase *adhA* gene were both induced in PAO1 Δ*metF* also in comparison to PAO1 after 12 h. The gene *uspK* is highly induced in PAO1 Δ*metF* indicating the increased need for the protein. UspK is essential for surviving specific anaerobic energy stress conditions such as pyruvate fermentation and anaerobic stationary phase (Boes *et al.*, 2008; Schreiber *et al.*, 2006). Both genes were also observed upregulated in the study of Alvarez-Ortega and Harwood (2007) and under microaerobic conditions in SCFM (Palmer *et al.*, 2007a). Moreover, the gene *micA* encoding for DNA mismatch repair was upregulated in PAO1 Δ*metF* vs. PAO1 after 4 h. It would be very interesting to obtain further inside views into the stress response of PAO1 Δ*metF* since it appeared to be altered.

The PQS biosynthesis was increased in PAO1 after 12 h. The *pqsBCD* operon genes were at least 2-fold upregulated as well as the *kynABU* operon, which converts tryptophan to the precursor of PQS biosynthesis anthranilic acid (Farrow and Pesci, 2007). Oxygen is necessary for the last step of PQS biosynthesis by PqsH (Schertzer *et al.*, 2010). The *antRABC* operon, which converts anthranilic acid to catechol, was also upregulated in PAO1 after 12 h. Both PQS synthesis and anthranilate degradation for energy production take place in PAO1. No increase or change of expression of PQS biosynthesis genes was observed for PAO1 Δ*metF*. The *kynABU* operon was downregulated in PAO1 Δ*metF* after 12 h, therefore the anthranilic acid pool is not refilled via tryptophan.

No difference in expression of the genes encoding the phenazine pyocyanin biosynthesis e.g. the *phzABCDEFG* operon, *phzM* and *phzS* was observed for both strains. The observed reduction of pyocyanin production in PAO1 Δ*metF* is not due to transcriptional downregulation. In addition, pyocyanin can be used to maintain a stable redox equilibrium. Pyocyanin can oxidise NADH to NAD^+ without the need for a catalysing enzyme during oxygen limitation in the culture as shown by a recent study (Dietrich *et al.*, 2013). PAO1 Δ*metF* possesses probably an altered redox balance compared to PAO1. This is probably indicated by a 2-fold upregulation of the expression of RoxS is in PAO1 Δ*metF* after 12h, which also might be reflected in the strong induction of cbb_3-type 2 oxidase operon.

Methionine and SAM metabolism genes are upregulated in *Pseudomonas aeruginosa* PAO1 Δ*metF*

The majority of the genes differently regulated in PAO1 and PAO1 Δ*metF* were genes encoding for proteins of the methionine and SAM metabolism (Table 18). These proteins are involved in methionine synthesis or are necessary for the conversion to SAM. SAM is used for various reactions. It is the second most frequently used enzyme substrate after ATP (Fontecave et al., 2004; Loenen, 2006). Approximately 95 % of SAM are used for methylation reactions (Griffith, 1987). The remaining SAH after a methylation reaction is recycled via SahH (Fig. 17). The corresponding gene *sahH* was 5.5-fold upregulated in PAO1 Δ*metF* and downregulated in PAO1 after 4 h. The SahH protein is a very stable enzyme. The upregulation of the *sahH* gene indicates the necessity and the high demand for the recycling of SAM via SahH in PAO1 Δ*metF*. This might indicate an unbalanced SAM /SAH level in PAO1 Δ*metF*.

The final step of methionine biosynthesis is the conversion of Hcy via either MetE or MetH to methionine. MetH is a cobalamine dependent and MetE a cobalamine independent methionine synthase. Under the investigated conditions in PAO1 Δ*metF* MetE was clearly the enzyme induced, since the *metE* gene was 168.3-fold and 59.5-fold upregulated, respectively. For *metH* only a 4.8-fold upregulation in PAO1 Δ*metF* after 4 h was observed. The gene *metE* shows the highest induction of all genes indicating the extraordinary need for methionine in PAO1 Δ*metF*. Both enzymes use the cofactor Me-THF or Me-THP as methyl-group donor, respectively. Me-THF or Me-THP is generated by MetF from N^5,N^{10}-methylene-THF or N^5,N^{10}-methylene-THP. This pathway is blocked by the deletion of *metF* in PAO1 Δ*metF*.

Methionine can be directly incorporated into biosynthesis or further converted via S-adenosylmethionine synthetase MetK to SAM. The *metK* gene was also upregulated in PAO1 Δ*metF* indicating the demand for SAM. The genes of the cobalamin and folate metabolism were identified upregulated in PAO1 Δ*metF* after 4 h. Cysteine metabolism genes were only upregulated in the PAO1 wt after 12 h.

The genes of the cleavage system *gcvT2H2P2*, *glyA1* hydroxy-methyltransferase and *metE* are described to be under positive quorum sensing control (Schuster *et al.*, 2003; Wagner *et al.*, 2003).

The need for methionine and SAM is also reflected by the upregulation of the transcriptional regulator gene *metR* in PAO1 Δ*metF* after 4 h and after 12 h. This is in accordance with *E. coli*, for which it is described that MetR is an activator of expression of the genes *metE, metH, glyA* and probably *metF* (Cowan *et al.*, 1993; Maxon *et al.*, 1989). Hcy acts as co-activator together with MetR for the expression of the genes *metE* and *glyA* or as co-repressor for *metH* expression (Plamann and Stauffer, 1989). In addition, MetR is regulated by MetJ in *E. coli*, which is regulating all methionine biosynthesis genes with exception of *metH* and *metK* according to the cellular SAM level (Greene, 1996). No MetJ homolog is annotated for *P. aeruginosa* so far and not much is known about the regulation of methionine and SAM metabolism genes in *P. aeruginosa*.

Table 18: Microarray analysis of selected methionine metabolism and SAM metabolism genes in PAO1 compared to PAO1 Δ*metF* in M9 0.5 % caseinate

Differentially regulated genes of *P. aeruginosa* PAO1 compared to PAO1 Δ*metF* after 4 h and after 12 h in M9 0.5 % caseinate under microaerobic to anaerobic conditions. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated of PAO1 wt 4 h vs. PAO1 Δ*metF* 4 h (W4 / M4), PAO1 wt 12 h vs. PAO1 Δ*metF* wt 12 h (W12 / M12), PAO1 Δ*metF* 12 h vs PAO1 Δ*metF* 4 h (M12 / M4) and PAO1 wt 12 h vs PAO1 wt 4 h (W12 / W4).

Gene ID[A]	Gene name[A]	Function[A]	FC			
			W4 / M4	W12 / M12	M12 / M4	W12 / W4
PA0314		L-cysteine transporter of ABC system FliY	2,4		-2,1	-4,5
PA0315		hypothetical protein		2,5	-3,2	-2,0
PA0316	*serA*	D-3-phosphoglycerate dehydrogenase	-15,5	-4,2	-2,2	
PA0390	*metX*	homoserine O-acetyltransferase			-2,7	
PA0430	*metF*	5,10-methylenetetrahydrofolate reductase	2,5	7,2		3,2
PA0431		hypothetical protein	-2,7			
PA0432	*sahH*	S-adenosyl-L-homocysteine hydrolase	-5,5			2,6
PA0546	*metK*	methionine adenosyltransferase	-5,4	-2,4		2,0
PA0547		probable transcriptional regulator	-8,6	-3,2		4,1
PA0582	*folB*	dihydroneopterin aldolase	-10,1		-3,6	
PA0583		7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase	-7,9		-3,6	
PA1276	*cobC*	cobalamin biosynthetic protein CobC	-2,1			2,3
PA1838	*cysI*	sulfite reductase				2,5
PA1843	*metH*	methionine synthase	-4,8		-3,8	
PA1927	*metE*	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	-168,3	-59,5	-2,1	
PA2445	*gcvP2*	glycine cleavage system protein P2				2,3
PA2446	*gcvH2*	glycine cleavage system protein H2				2,5
PA3169	*mtnA*	5-methylthioribose-1-phosphate isomerase MtnA				2,2
PA3587	*metR*	transcriptional regulator MetR	-3,3	-3,1		
PA3816	*cysE*	O-acetylserine synthase				2,3
PA3824	*queA*	S-adenosylmethionine:t-Rna ribosyltransferase-isomerase				2,1
PA4314	*purU1*	formyltetrahydrofolate deformylase			-2,2	
PA4442	*cysN*	ATP sulphurylase GTP-binding subunit/APS kinase				3,1
PA4443	*cysD*	ATP sulphurylase small subunit				3,5
PA4602	*glyA3*	serine hydroxymethyltransferase	-12,8	-2,4	-2,6	2,1
PA5213	*gcvP1*	glycine cleavage system protein P1	2,4			-3,5
PA5415	*glyA1*	serine hydroxymethyltransferase	2,5			-2,5

[A] Gene ID, gene name, function and PseudoCAP Function Class (PCFC) are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

For PA14 was shown that the deletion of *metR* abolished the ability to swarm (Yeung *et al.*, 2009). Moreover, they detected that *metH* and *metF* were downregulated in PA14 *metR*$^-$ mutant. They also observed a downregulation of virulence-associated genes under the tested swarming conditions. One can assume that part of the regulatory network for the regulation of methionine biosynthesis genes e.g. *metH* and *metF* is similar to *E. coli*. As mentioned several methionine and SAM biosynthesis genes are also quorum sensing regulated (Heurlier *et al.*, 2006; Schuster *et al.*, 2003; Wagner *et al.*, 2003). But since no MetJ regulator was identified for *P. aeruginosa* the regulatory network, especially concerning the regulation of virulence factors and in regard to quorum sensing regulation might be totally different.

In addition, a recent study described the presence of a SAH dependent riboswitch in the mRNA of the *sahH* operon for *Pseudomonas syringae* (Wang *et al.*, 2008). Wang *et al.* proposed that SAH binding activates the gene expression. 15 SAH RNA motifs were identified in the Pseudomonadales (Wang *et al.*, 2008). Recently, a SAM-III box was identified in the 5´untranslated region (5´ UTR) of *metK* in *Enterococcus faecalis* (Smith *et al.*, 2010). The presence of SAH or SAM dependent riboswitches in the 5´UTR of *sahH*, *metK* and *metH* seemed possible from the results of β-galactosidase assays (4.2.10) for *P. aeruginosa* PAO1.

Sequence analysis (4.2.9) neither proved nor disproved this assumption nor did the here conducted microarray analysis. The regulatory network of *P. aeruginosa* methionine and SAM metabolism has to be further analysed to identify the underlying regulatory network and its regulators.

Methionine metabolism - a connection point for iron metabolism in *Pseudomonas aeruginosa*

A connection between iron and sulphur metabolism can be assumed. In this study several iron starvation genes were detected upregulated in PAO1 Δ*metF* after 12 h. The probable ECF sigma factor PA1300 and the probable membrane sensor PA1301 were upregulated in PAO1 Δ*metF*, respectively (Table 19).

Moreover, the genes encoding for the biosynthesis of the siderophores pyoverdine and pyochelin were upregulated. The regulator *pvdS* gene was downregulated in PAO1 Δ*metF* compared to PAO1 after 12 h. PvdS expression is described to be oxygen dependent (Vasil, 2007), but after 12 h both cultures were oxygen depleted (Fig. 23 C). A previous study showed that CysB the central regulator of sulphur metabolism in *P. aeruginosa* is crucial for PvdS expression (Imperi *et al.*, 2010). The *cysB* gene (PA1754) was found downregulated in the PAO1 after 12 h and was not detected in the PAO1 Δ*metF* (Appendix Table 34).

Another well-known connection between iron and sulphur metabolism are the iron-sulphur clusters, which are essential prosthetic groups for electron transfer, gene regulation and environmental sensing. Therefore, assembly is carefully regulated to avoid toxicity of free iron and sulphide (Ayala-Castro *et al.*, 2008). On the other hand energy is saved, when uptake and assembly are coregulated. One can discuss if an unbalanced methionine and SAM level might also influence indirectly iron metabolism and uptake.

A previous study examined the influence of MetR on the virulence of *Aspergillus fumigatus*. They identified MetR as potential regulator of the sulphur assimilation, especially of inorganic sources (Amich *et al.*, 2013). The *A. fumigatus* Δ*metR* strain was attenuated in virulence in two model systems, but could be complemented by methionine.

A. fumigatus Δ*metR* strain showed an increased intracellular iron content, while showing the gene expression pattern of iron starvation (Amich *et al.*, 2013). The intracellular iron content was even increased in the absence of methionine and even more iron was present chelated by ferricrocin in Δ*metR* with and without methionine supplementation. They observed an upregulation of iron uptake genes under sulphur starvation conditions. Furthermore, they detected a hypersensitivity of the *A. fumigatus* Δ*metR* towards iron. In the presence of high iron concentrations the *A. fumigatus* Δ*metR* was unable to grow.

One can assume that iron sulphur homeostasis might also be affected in PAO1 Δ*metF*. The effect might not be as strong as in a *A. fumigatus* Δ*metR* mutant, as *metF* is not a transcriptional regulator but an enzyme of methionine and SAM metabolism. Furthermore, the regulatory network of regulator MetR in *P. aeruginosa* is probably different from *A. fumigatus*.

Table 19: Microarray analysis of selected iron regulated genes in PAO1 compared to PAO1 Δ*metF* in M9 0.5 % caseinate

Differentially regulated genes of *P. aeruginosa* PAO1 compared to PAO1 Δ*metF* after 4 h and after 12 h in M9 0.5 % caseinate under microaerobic to anaerobic conditions. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated of PAO1 wt 4 h vs. PAO1 Δ*metF* 4 h (W4 / M4), PAO1 wt 12 h vs. PAO1 Δ*metF* wt 12 h (W12 / M12), PAO1 Δ*metF* 12 h vs PAO1 Δ*metF* 4 h (M12 / M4) and PAO1 wt 12 h vs PAO1 wt 4 h (W12 / W4).

Gene ID[A]	Gene name[A]	Function[A]	FC W4 / M4	FC W12 / M12	FC M12 / M4	FC W12 / W4
Iron starvation:						
PA0346		hypothetical protein			2,6	
PA0471		probable transmembrane sensor			2,1	
PA0472	*fiuI*	probable sigma-70 factor, ECF subfamily			2,6	3,5
PA0672	*hemO*	heme oxygenase			2,7	4,0
PA0707	*toxR*	transcriptional regulator ToxR			2,7	3,0
PA0929		two-component response regulator				2,0
PA0930		two-component sensor				2,2
PA1134		hypothetical protein			5,7	3,4
PA1300		probable sigma-70 factor, ECF subfamily		-2,2	11,8	8,4
PA1301		probable transmembrane sensor		-2,3	7,9	4,6
PA2033	*viuB*	hypothetical protein			2,9	2,2
PA2034		hypothetical protein		-2,3	7,8	4,1
PA2385	*pvdQ*	3-oxo-C12-homoserine lactone acylase PvdQ			18,8	18,8
PA2386	*pvdA*	L-ornithine N5-oxygenase				2,7
PA2388	*fpvR*	FpvR			4,6	4,3
PA2391	*opmQ*	probable outer membrane protein precursor			10,5	8,8
PA2392	*pvdP*	PvdP			5,3	6,0
PA2393		probable dipeptidase precursor			7,2	7,9
PA2394	*pvdN*	PvdN			2,9	3,1
PA2395	*pvdO*	PvdO			9,1	10,5
PA2396	*pvdF*	pyoverdine synthetase F			6,2	6,9
PA2397	*pvdE*	pyoverdine biosynthesis protein PvdE			2,0	3,7
PA2398	*fpvA*	ferripyoverdine receptor			18,7	12,9
PA2399	*pvdD*	pyoverdine synthetase D			15,5	8,7
PA2400	*pvdJ*	PvdJ			14,8	9,1
PA2402		probable non-ribosomal peptide synthetase			13,5	10,9
PA2404		hypothetical protein			2,4	2,9
PA2405		hypothetical protein			2,3	2,5
PA2406		hypothetical protein			2,4	2,4
PA2407		probable adhesion protein			2,2	2,6
PA2408		probable ATP-binding component of ABC transporter			3,4	3,0
PA2411		probable thioesterase		-2,0	21,1	15,1
PA2412		conserved hypothetical protein			17,3	17,9
PA2413	*pvdH*	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase		-2,1	14,4	8,3
PA2424	*pvdL*	PvdL		-2,1	21,6	11,4
PA2425	*pvdG*	PvdG			8,9	7,6
PA2426	*pvdS*	sigma factor PvdS		2,2	2,6	7,6
PA2427		hypothetical protein			23,9	14,2
PA2451		hypothetical protein			2,8	
PA2467	*foxR*	Anti-sigma factor FoxR			3,7	2,9
PA2468	*foxI*	ECF sigma factor FoxI				2,5
PA3285		probable sigma-70 factor, ECF subfamily				2,3
PA3397	*fpr*	ferredoxin-NADP+ reductase				4,3
PA3407	*hasAp*	heme acquisition protein HasAp			4,2	2,3
PA3530		conserved hypothetical protein			2,2	4,7
PA3786		hypothetical protein			2,0	
PA4220		hypothetical protein			7,3	6,9
PA4221	*fptA*	Fe(III)-pyochelin outer membrane receptor precursor			4,1	4,5
PA4222		probable ATP-binding component of ABC transporter			6,4	5,1
PA4223		probable ATP-binding component of ABC transporter			7,1	6,2
PA4224	*pchG*	pyochelin biosynthetic protein PchG		-2,0	8,5	7,5
PA4225	*pchF*	pyochelin synthetase			7,5	7,6
PA4226	*pchE*	dihydroaeruginoic acid synthetase			6,4	6,5
PA4227	*pchR*	transcriptional regulator PchR			2,2	2,4
PA4228	*pchD*	pyochelin biosynthesis protein PchD			4,4	4,1
PA4229	*pchC*	pyochelin biosynthetic protein PchC			8,0	4,4
PA4230	*pchB*	salicylate biosynthesis protein PchB			5,6	4,3
PA4231	*pchA*	salicylate biosynthesis isochorismate synthase			8,2	5,5
PA4236	*katA*	catalase				-2,5

Continued:

Gene ID[A]	Gene name[A]	Function[A]	FC W4 / M4	FC W12 / M12	FC M12 / M4	FC W12 / W4
PA4358		probable ferrous iron transport protein		-4,4	8,7	
PA4359		conserved hypothetical protein		-3,1	5,8	2,1
PA4362		hypothetical protein				-3,2
PA4366	*sodB*	superoxide dismutase		2,5	-3,6	
PA4370	*icmP*	Insulin-cleaving metalloproteinase outer membrane pp.*		2,0		
PA4467		hypothetical protein		-3,5	14,2	3,7
PA4468	*sodM*	superoxide dismutase		-2,5	6,3	2,5
PA4469		hypothetical protein		-3,1	11,1	3,4
PA4470	*fumC1*	fumarate hydratase		-2,8	13,4	4,6
PA4471		hypothetical protein			5,3	3,4
PA4570		hypothetical protein			11,9	5,4
PA4708	*phuT*	Heme-transport protein, PhuT			3,0	2,9
PA4709		probable hemin degrading factor			3,1	3,6
PA4895		probable transmembrane sensor			2,7	
PA4896		probable sigma-70 factor, ECF subfamily			2,2	
PA5150		probable short-chain dehydrogenase			4,1	
Regulated by PA4896[B] (all genes classified as "related to phage, transposon, or plasmid"):						
PA0614		Putative holin			3,5	2,9
PA0615		R-type pyocin, related to P2 phage		-2,0	3,4	2,5
PA0616		R-type pyocin, related to P2 phage; tail spike			3,5	2,1
PA0617		R-type pyocin, related to P2 phage; baseplate			4,3	2,4
PA0618		R-type pyocin, related to P2 phage; baseplate/tail fibre			4,3	2,5
PA0619		R-type pyocin, related to P2 phage; tail formation			4,1	2,1
PA0620		R-type pyocin, related to P2 phage; tail fibre			3,3	2,1
PA0621		R-type pyocin, related to P2 phage; tail fibre assembly			2,3	
PA0622		R-type pyocin, related to P2 phage; tail sheath			4,8	2,4
PA0623		R-type pyocin, related to P2 phage; tail tube			4,1	2,0
PA0624		R-type pyocin, related to P2 phage			4,8	
PA0625		R-type pyocin, related to P2 phage; tail length det.*			3,0	2,0
PA0626		R-type pyocin, related to P2 phage; tail formation			2,7	
PA0627		R-type pyocin, related to P2 phage; tail formation			3,9	2,1
PA0628		R-type pyocin, related to P2 phage; tail formation			3,0	2,2
PA0629		Related to phage, lytic enzyme			2,3	
PA0630		Related to phage, lysis control			3,3	
PA0631		Related to phage, lysis control			3,2	2,1
PA0633		F-type pyocin, related to l phage; major tail protein			3,1	
PA0634		F-type pyocin, related to l phage			4,4	2,1
PA0635		F-type pyocin, related to l phage			3,6	2,1
PA0636		F-type pyocin, related to l phage; tail length determination			4,9	3,2
PA0637		F-type pyocin, related to l phage; tail formation			4,4	2,3
PA0638		F-type pyocin, related to l phage; tail formation			4,5	2,9
PA0639		PF-type pyocin, related to l phage; tail formation		-2,1	3,9	
PA0640		probable bacteriophage protein			2,2	
PA0641		probable bacteriophage protein			2,0	
PA0644		F-type pyocin, related to l phage			2,2	
PA0645		F-type pyocin, related to l phage			2,4	
Predicted Fur regulation by (van Oeffelen *et al.*, 2008):						
PA5504		D-methionine ABC transporter membrane protein				2,1
PA4296	*pprB*	two-component response regulator, PprB	2,7		-2,1	-5,6

[A] Gene ID, gene name, function and PseudoCAP Function Class (PCFC) are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

[B] Functions for genes regulated by PA4896 are annotated according to (Llamas *et al.*, 2008)

Abbreviations: det. (determination); pp (protein precursor)

3.3 Conclusions

This study confirms the importance of methionine auxotrophy among the clinical *P. aeruginosa* CF strains. In total 13 different clone types among the 15 patients and the 30 methionine auxotrophic strains were identified.

This study is the first to determine the genetic cause of methionine auxotrophy. 14 strains were complemented with the *metF* (PA0430) gene. PA0430 encodes for a 5,10-methylene tetrahydrofolate reductase. Moreover, two additional strains contained a mutation in *metF* gene and an additional mutation in the *metH* (PA1843) gene. The sequence analysis did not reveal a mutational hot spot within the *metF* gene. The growth of the remaining 14 non-complemented methionine auxotrophic strains was only complemented by methionine, which indicates that the mutation must be in the genes of the cofactor biosynthesis, *metE* and/or *metH* or a regulator.

The *metF* mutation, itself does not seem to enhance the mutator frequency since PAO1 Δ*metF* did not show an increased mutator frequency compared to PAO1. This indicates that the methionine auxotrophy is selected independently of the mutator phenotype.

An important characteristic of the PAO1 Δ*metF* strain and 30 methionine auxotrophic clinical isolates was the reduced to abolished pyocyanin production. The transcriptom of PAO1 Δ*metF* did not indicate any alterations in phenazine biosynthesis gene expression. Therefore, the reduction of pyocyanin biosynthesis must be a direct effect of the *metF* mutation.

In general, methionine auxotrophic strains such as PAO1 Δ*metF* are dependent on external methionine, to refill their methionine pool and thereby refilling the SAM pool to be able to grow. On can discuss that decreased cellular methionine level together with decreased recycling of methionine most likely results in an imbalance of the intracellular SAM / SAH level, which is then influencing SAM dependent reactions such as e.g. pyocyanin biosynthesis or the transcription of the genes encoding for the iron-sulphur cluster containing radical SAM enzyme HemN and the SAM-dependent methylase NirE.

Antipseudomonal treatments target actively growing cells, slower or non-growing cells have a survival advantage. One can suspect that this might be also be true for *metF* mutation especially in clinical *P. aeruginosa* CF strains. During methionine depletion a strain carrying a *metF* mutation would be unable to grow. Depending on the degree of methionine availability strains would produce wt amounts of pyoverdin and reduced amounts of pyocyanin or act as cheater during methionine depletion and use pyoverdin and pyocyanin produced by other strains. With sufficient methionine supplementation odDHL and BHL production was even increased in PAO1 Δ*metF.* Thereby, a *metF* mutant might stimulate other strains to produce public goods such as pyoverdin and pyocyanin. This overproduction was abolished in all clinical methionine auxotrophic strains investigated. Strains carrying a *metF* mutation might also be able to accumulate more iron - one of the essential goods during infection. But this hypothesis has to be verified by further investigations of the intracellular iron content of PAO1 Δ*metF*.

3.4 Material and Methods

3.4.1 Isolation of *Pseudomonas aeruginosa* strains from the respiratory tract of cystic fibrosis patients

The samples from the respiratory tract of the CF patients were taken during routine examinations. Strains were isolated as described (Hogardt *et al.*, 2006). Strains were analysed for growth on minimal media and for growth on minimal media containing methionine. The strains were obtained from Michael Hogardt.

For the further analysis of the methionine auxotrophic clinical *P. aeruginosa* strains M9 20 mM succinate minimal medium (Sambrook and Russell, 2001) was used containing 1 x M9- salts [47.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl], 20 mM succinate, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 x trace metals [0.148 mg/l $ZnSO_4$ x 6 H_2O, 0.100 mg/l $MnCl_2$ x 4 H_2O, 0.236 mg/l $CoSO_4$ x 7 H_2O, 0.100 mg/l $NiCl_2$ x 6 H_2O, 0.020 mg/l $CuCl_2$ x 2 H_2O, 0.050 mg/l Na_2MoO_4 x 2 H_2O, 1 µl/l 25 % HCl modified after (Schlegel, 2007)] with and without 50 µM methionine (Sigma), respectively.

Besides, the clinical *P. aeruginosa* strains were investigated for growth on M9 succinate with supplementation of 50 µM L-serine, L-homoserine, O-succinyl-L-homoserine, L-cysteine or Hcy (all obtained from Sigma), respectively. Hcy and vitamin B12 were used at 100 µM, as described previously (Barth *et al.*, 1998).

3.4.2 Bacterial strains and growth conditions

All bacterial strains used in this study are shown in Table 20 and plasmids in Table 21. *E. coli* and *P. aeruginosa* strains were grown routinely in LB medium for standard molecular biology protocols. For *P. aeruginosa* the following concentrations of antibiotics were used: carbenicillin 250 µg/ml, gentamicin 80 µg/ml and tetracycline 100 µg/ml. The *E. coli* strain DH10B was used as a host for cloning and ST18 for transfer of plasmids and cosmids to *P. aeruginosa*. The *E. coli* strain JM109, which was used for the biolumescence assay for the determination of AHL concentration, was incubated at 30°C.

Cultures were inoculated at OD_{578} of 0.05 and shaken at 200 rpm at 37°C, if not indicated otherwise. For growth experiments in MHB an over night culture in LB medium was prepared.

For growth experiments in M9 0.5% caseinate an over night culture in amino acid medium modified (AAMM) was prepared. AAMM (pH 6.9) contains 86 mM NaCl, 29 mM KCl, 20 mM Hepes, 15 µM DTPA, 1.75 mM $CaCl_2$, 0.61 mM $MgCl_2$, 0.271 mM $MgSO_4$ and 2.55 mM Na_2HPO_4/ NaH_2PO_4 (pH 6.9) and 19 mM amino acids [1.8 mM alanine, 1.6 mM glutamate, 1.1 mM isoleucine, 0.01 mM tryptophan, 1.6 mM leucine, 0.8 mM aspartate, 1.7 mM proline, 2.1 mM lysine, 1.5 mM serine, 0.5 mM phenylalanine, 0.8 mM tyrosine, 1.1 mM valine, 1.1 mM threonine, 0.2 mM cysteine, 0.3 mM arginine, 1.2 mM glycine, 0.5 mM histidine (Sigma)]. The pH was adjusted with HCl and NaOH. For solid medium 15 g/l agar was added. Methionine was added in different concentrations depending on the experiment of 630 µM for simulated respiratory tract conditions or 100 µM or 200 µM.

3.4.3 Sequence analysis of the *metF* gene

Colony PCR was performed using the respective primers. The PCR product was gel purified using gel purification Kit (Qiagen) according to the manufacturer´s instructions. The sequence was determined by GATC. The sequence was analysed using the program 4Peaks.

3.4.4 Construction of *Pseudomonas aeruginosa* deletion strains

To obtain unmarked gene deletion mutants the well-established strategies based on *sacB* counterselection and FLP recombinase excision (Hoang *et al.*, 1998) were used. To achieve deletion of *mtnP* (PA3004) the pAW03 suicide plasmid, of *metZ* (PA3107) the pMR01 suicide plasmid, of *metE* (PA1927) the pFH06 suicide plasmid and of *metH* (PA1843) the pFH07 suicide plasmid was constructed.

For the construction of all plasmids the gentamycin resistance cassette of pPS858 was digested with the indicated enzymes (Table 22) and was cloned between two PCR fragments of the respective genes in the multiple cloning site of pEX18Ap, respectively. The two PCR fragments contained DNA homologous to upstream und downstream fragments of the above-mentioned genes, respectively, which should be deleted in the respective mutant strains. Primer sequence and vector details are shown in Table 22 and Table 21.

The construction of PAO1 Δ*sahH* and PAO1 Δ*metF* are described elsewhere (Thoma, 2009). The gene deletion constructs were introduced into *E. coli* DH10B by transformation. Plasmids containing the correct insert were transferred via diparental mating using *E. coli* strain ST18 (Thoma and Schobert, 2009) in *P. aeruginosa* PAO1. After successful gene deletion the gentamicin resistance cassette was removed from the genome using the pFLP2 plasmid to obtain unmarked gene deletion mutants (Hoang *et al.*, 1998). The resulting deletion mutants were verified by PCR. Double or triple deletion mutants were obtained by repeating the described procedure for each gene to be deleted (Table 20).

3.4.5 Construction of complementation vector and complementation of methionine auxotrophic *Pseudomonas aeruginosa* strains

To identify if the methionine auxotrophy in the clinical CF isolates was caused by a mutation in the *metF* gene (PA0430) a in total 872 bp PCR product, was amplified and cloned into pUCP20T (pST10) (Thoma, 2009). Some strains were also complemented by pFH04 (pUCP20T::*metF*::*metH*). Primer sequences and vector details are shown in Table 22 and Table 21. The transfer of pST10 and pFH04 plasmid in *P. aeruginosa* and clinical isolates was carried out as described previously (Boes *et al.*, 2008) modified by the use of ST18 (Thoma and Schobert, 2009).

3.4.6 Motility assays - swimming, swarming and twitching motility

Motility assays were performed as described, previously with minor modifications (O'Toole and Kolter, 1998; Yeung *et al.*, 2009). Briefly, twitching motility was determined in 1% LB agar. 2 µl of the investigated strains were inoculated between the agar and the petri dish and incubated for 24 h at 37°C and for 48 h at 25°C. Swimming motility was investigated in 0.3 % LB agar. 2 µl

of the investigated strains were inoculated into the middle of the agar and incubated for 24 h at 37°C.

For the investigation of swarming motility a medium containing 0.5 % agar, 1 x M8- salts [47.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl], 0.4% glucose, 0.5 % casamino acids, 2 mM $MgSO_4$ and 10 µM $FeSO_4$ was used. 2 µl of the investigated strains were inoculated on the agar surface and incubated for 48 h at 37°C. Hazy zones around the inoculation were measured and the radius [mm] determined.

3.4.7 AHL quorum sensing molecule formation

For the determination of AHL molecule formation of the clinical isolates KI-AW01 to KI-AW28, CFCZ, PAO1 and PAO1 Δ*metF* the strains were grown over night in LB and inoculated to OD_{578} 0.05 in 10 ml Mueller-Hinton broth (MHB; Fluka). Cultures were shaken at 200 rpm at 37°C. After 24 h OD_{578} was measured.

For the investigation of quorum sensing molecule formation of methionine metabolism deletion strains, the strains were grown over night in AAMM and inoculated to OD_{578} 0.05 in 10 ml M9 0.5% caseinate. Cultures were shaken at 200 rpm at 37°C. After 24 h OD_{578} was measured.

Samples were prepared as described previously with minor modification of reduced sample size of 100 µl and incubation at 17°C for 18 h (Diggle *et al.*, 2002).

The *E. coli* JM109 strains harbouring the plasmids pSB1075 or pSB406 were used for bioluminescence assay in order to determine odDHL or BHL concentration, respectively. The assay was performed as described, previously (Winson *et al.*, 1998). Briefly, 2 µl acidified and diluted sample or reference BHL (Fluka) or odDHL (Sigma) were mixed with 200 µl *E. coli* culture in a 96 well luminescence plate (Greiner) and sealed with sealing film Breath-easy (Roth) for gas exchange and shaken at 600 rpm (Varioskan Flash, Thermo Scientific) at 30°C for 2 h, during incubation OD_{578} and bioluminescence was measured.

AHL concentrations were calculated according to the respective calibration curves determined for each measurement. odDHL was diluted in DSMO and BHL was diluted in MilliQ water. End concentrations of 0.63 nM to 80 nM odDHL and of 15.25 nM to 3.9 µM BHL in 200 µl were used for calibration.

3.4.8 Mutator phenotype

To investigate the frequency of mutator phenotype among the clinical CF isolates the strains cultures were inoculated at OD_{578} of 0.05 in 10 ml MHB and shaken at 200 rpm at 37°C for 24 h. CFU was determined on LB agar and mutator frequency was determined on LB rifampicin 100 µg / ml (RA-100). Mutator frequency was calculated CFU RA-100 resistant bacteria vs. LB CFU. We modified the terms defined by Ciofu *et al.* (2010). All strains with a mutator frequency ≥ 1.5×10^{-7} were classified as strong mutators. Strains with lower mutator frequency of < 1.5×10^{-7} to ≥ 1.5×10^{-8} were described as weak mutators. A mutator frequency of < 1.5×10^{-8} similar to the *P. aeruginosa* PAO1 was considered as non-mutator. *P. aeruginosa* PAO1 Δ*mutS* was used as positive control (Hogardt *et al.*, 2006).

3.4.9 Virulence factor production - pyocyanin and pyoverdin

For virulence factor production experiments, *P. aeruginosa* PAO1 and several methionine metabolism deletion mutants were grown in M9 minimal medium 0.5% caseinate (Sambrook and Russell, 2001) containing 1 x M9- salts [47.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl], 0.5% caseinate, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 x trace metals [0.148 mg/l $ZnSO_4$ x 6 H_2O, 0.100 mg/l $MnCl_2$ x 4 H_2O, 0.236 mg/l $CoSO_4$ x 7 H_2O, 0.100 mg/l $NiCl_2$ x 6 H_2O, 0.020 mg/l $CuCl_2$ x 2 H_2O, 0.050 mg/l Na_2MoO_4 x 2 H_2O, 1 µl/l 25 % HCl modified after (Schlegel, 2007)], respectively. Cultures were inoculated at OD_{578} of 0.05 in 10 ml medium and shaken at 200 rpm at 37°C, if not indicated otherwise.

OD_{578} was determined after 24 h and 1 ml supernatant was sterilised by filtration. Pyocyanin and pyoverdin was extracted as described, previously. Pyocyanin was determined at OD_{695} and pyoverdin fluorescence was excited at 405 nm and emission was determined as relative fluorescence F_{460}. As negative control for the pyocyanin production *P. aeruginosa* PA14 Δ*phz* was used. The results of at least three independent experiments are shown.

3.4.10 Transcriptome analysis in M9 0.5% caseinate and measurement of oxygen concentration

PAO1 and PAO1 Δ*metF* were cultivated in 250 ml M9 0.5% caseinate in 300 ml shaking flaks without baffles at 37°C and 200 rpm. The over night culture was prepared in AAMM with 630 µM methionine. We used Fibox 3 LCD-trace (PreSens) together with the autoclavable oxygen sensor spot SP-PSt3 (PreSens), which was permanently attached inside the shaking flask for oxygen concentration measurement [µmol/l]. Data were exported with OxyView - LCDTRACE-V2.04 10/2009 (PreSens). OD_{578} was measured using a photometer Ultrospec (Amersham Biosciences).

3.4.11 RNA isolation, RNA enrichment and cDNA amplification

For RNA isolation 25 ml of bacterial culture were harvested at the respective time points after 4 h and 12 h. The sample was incubated with 15 ml RNAprotect® Bacteria Reagent (Qiagen) according to the manufacturer's instructions. Samples were stored at - 80°C. RNA was isolated using RNeasy® Mini Kit (Qiagen). The RNA isolation was performed as described previously with minor modifications (Bielecki *et al.*, 2011). Bacteria were lysed using 400 µl TE buffer containing 1 mg/ml lysozyme with periodic vortexing every 2 min for 15 sec for 10 min. 700 µl RLT puffer containing 1% β-mercaptoethanol were added. Afterwards samples were centrifuged at maximal speed (14.100 x g) for 2 min. The same amount of ethanol was added to the samples. The samples were loaded to a spin column, washed with RW1 buffer afterwards DNA was digested using RNase-free DNase I. RNA was eluted in 50 µl and 30 µl of RNase-free water. Eluted RNA was treated a second time with DNase I to ensure that all traces of genomic DNA were removed. The isolated RNA was stored at – 80°C. The yield of the isolated RNA was measured by light absorption at 260 nm using a Nanodrop ND-1000-UV/VIS (Peqlab). Integrity and purity was checked by 2100 BioAnalyzer (Agilent Technologies).

P. aeruginosa GeneChip from Affymetrix requires 7,5 - 10 µg of RNA per microarray. Bacterial RNA amplification was used. This was performed using the MessageAmp Bacteria Kit®

(Ambion). The first step was to treat the total enriched bacterial RNA with a polyadenylation polymerase to produce polyA tails. The single stranded cDNA was produced using reverse transcriptase and oligo dT primers. Afterwards a second strain cDNA was produced, and the double stranded cDNA served as a template for in vitro transcription using T7 RNA polymerase and T7 oligonucleotides. During the in vitro transcription reaction the modified nucleotides biotin-11-CTP (PerkinElmer Life Sciences) and biotin-16-UTP (Roche Applied Science) were used. As a result antisense biotinylated RNA ready to use for GeneChip® hybridization was obtained.

3.4.12 Microarray hybridisation

Amplified RNA is commonly used for eukaryotic microarrays, but the original procedure for *P. aeruginosa* Gene-Chip® (Affymetrix) was for single stranded terminally labelled cDNA. As described previously, no difference in results for microarray analysis were observed for the standard procedure (without amplification) RNA procession or amplified RNA (Francois *et al.*, 2007). The hybridization and washing steps were performed in the Affymetrix Array facility at Helmholtz Centre for Infection Research (HZI) Braunschweig (Dr. Robert Geffers). Since the RNA had been amplified, some changes were introduced to the original Affymetrix protocol, such as that the RNA was fragmented using 5 x fragmentation buffer instead of DNase I treatment. Hybridisation was performed as described previously (Bielecki *et al.*, 2011).

3.4.13 Microarray data analysis

Raw microarray data were pre-processed with the Bioconductor software framework (Gentleman *et al.*, 2004). Expression values were calculated as described, previously (Trunk *et al.*, 2010). Robust Multichip Average (RMA) method using quantile normalization, background corrected PM intensities and median polish as summarization method were applied (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003a; Irizarry *et al.*, 2003b).

In addition, the pdes were computed for the following pairwise comparison of experimental conditions: PAO1 wt after 4 h versus PAO1 wt after 12 h, PAO1 Δ*metF* after 4 h versus PAO1 Δ*metF* after 12 h, PAO1 wt after 4 h versus PAO1 Δ*metF* after 4 h and PAO1 wt after 12 h versus PAO1 Δ*metF* after 12 h. For this purpose, the pre-processed expression data were analysed by a regularized t-test based on a Bayesian statistical framework using the CyberT algorithm (Baldi and Long, 2001; Hatfield *et al.*, 2003). The R package bayesreg, which implements the CyberT algorithm, was used for computing the posterior probabilities of differential expression (free download under http://cybert.microarray.ics.uci.edu/). The mean gene expression levels of the replicate experiments were ranked in ascending order, a sliding window of 101 genes was used and a confidence value of 10 was chosen as weight. More information on the use of this regularized t-test in the context of determining differentially expressed genes of prokaryotic DNA microarray expression data is published elsewhere (Hung *et al.*, 2002). A combination of information from expression ratios and pdes allowed to identify the genes with the highest relative changes in expression values and the highest probabilities of differential expression at the same time.

Table 20: Bacterial strains

Bacterial strains	Genotype / description	Reference
P. aeruginosa:		
PAO1	Wild type	(Dunn and Holloway, 1971)
PAO1 Δ*mutS*	PAO1 Δ*mutS* (PA3620)	(Hogardt *et al.*, 2006)
ST30	PAO1 Δ*metF* (PA0430)	(Thoma, 2009)
ST26	PAO1 Δ*sahH* (PA0432)	(Thoma, 2009)
AW07	PAO1 Δ*mtnP* (PA3004)	This study
AW12	PAO1 Δ*sahH* Δ*mtnP* (PA0432, PA3004)	This study
AW18	PAO1 Δ*metZ* (PA3107)	This study
FH01	PAO1 Δ*metH* (PA1843)	This study, Frederike Haack
FH03	PAO1 Δ*metE* (PA1927)	This study, Frederike Haack
FH05	PAO1 Δ*metE* Δ*metH* (PA1927, PA1843)	This study, Frederike Haack
FH08	PAO1 Δ*metE* Δ*metF* Δ*metH* (PA1927, PA0430, PA1843)	This study, Frederike Haack
AW20	PAO1 pUCP20T	This study
AW21	PAO1 Δ*metF* pST10	This study
PA14 Δ*phz*	PA14 Δ*phzA1-G1* Δ*phzA2-G2*	(Dietrich *et al.*, 2006)
Clinical cystic fibrosis isolates:		
CFCZ	Clinical cystic fibrosis isolate from patient A, Medizinische Hochschule Hannover (MHH)	(Bielecki *et al.*, 2013)
CF-1	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
CF-2	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
CF-3	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
CF-4	Clinical cystic fibrosis isolate from patient B	(Bielecki *et al.*, 2013)
KI-AW 1	Clinical cystic fibrosis isolate from patient 1	Michael Hogardt
KI-AW 2	Clinical cystic fibrosis isolate from patient 2	Michael Hogardt
KI-AW 3	Clinical cystic fibrosis isolate from patient 2	Michael Hogardt
KI-AW 4	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 5	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 6	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 7	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 8	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 9	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 10	Clinical cystic fibrosis isolate from patient 4	Michael Hogardt
KI-AW 11	Clinical cystic fibrosis isolate from patient 4	Michael Hogardt
KI-AW 12	Clinical cystic fibrosis isolate from patient 5	Michael Hogardt
KI-AW 13	Clinical cystic fibrosis isolate from patient 5	Michael Hogardt
KI-AW 14	Clinical cystic fibrosis isolate from patient 6	Michael Hogardt
KI-AW 15	Clinical cystic fibrosis isolate from patient 6	Michael Hogardt
KI-AW 16	Clinical cystic fibrosis isolate from patient 7	Michael Hogardt
KI-AW 17	Clinical cystic fibrosis isolate from patient 8	Michael Hogardt
KI-AW 18	Clinical cystic fibrosis isolate from patient 8	Michael Hogardt
KI-AW 19	Clinical cystic fibrosis isolate from patient 8	Michael Hogardt
KI-AW 20	Clinical cystic fibrosis isolate from patient 9	Michael Hogardt
KI-AW 21	Clinical cystic fibrosis isolate from patient 3	Michael Hogardt
KI-AW 22	Clinical cystic fibrosis isolate from patient 10	Michael Hogardt
KI-AW 23	Clinical cystic fibrosis isolate from patient 8	Michael Hogardt
KI-AW 24	Clinical cystic fibrosis isolate from patient 8	Michael Hogardt
KI-AW 25	Clinical cystic fibrosis isolate from patient 11	Michael Hogardt
KI-AW 26	Clinical cystic fibrosis isolate from patient 6	Michael Hogardt

Continued:

Bacterial strains	Genotype / description	Reference
KI-AW 27	Clinical cystic fibrosis isolate from patient 6	Michael Hogardt
KI-AW 28	Clinical cystic fibrosis isolate from patient 12	
65	Clinical cystic fibrosis isolate from patient C	Gerd Döring, Tübingen
BT72	Clinical cystic fibrosis isolate from patient D	MHH
E. coli:		
DH10B	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu) 7697 galU galK λ⁻ rpsL nupG*	GibcoBRL (Invitrogen)
ST18	S17 λ*pir* Δ*hemA*	(Thoma and Schobert, 2009)
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA [FP traD36 proAB lacI lacZ ΔM15]* with pSB1075 (for odDHL detection) or with pSB406 (for BHL detection)	(Yanisch-Perron *et al.*, 1985) (Winson *et al.*, 1998)

Table 21: Plasmids

Plasmid	Description	Reference
pEX18AP	Ap^r, *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS of pUC18	(Hoang *et al.*, 1998)
pPS858	Ap^r, Gm^r, source of gentamicin cassette	(Hoang *et al.*, 1998)
pFLP2	Ap^r, source of FLP recombinase	(Hoang *et al.*, 1998)
pUCP20T	Cb^r, mobilizable *E. coli - P. aeruginosa* shuttle vector	(Schweizer, 1996)
pST06	Ap^r Gm^r; pEX18Ap with 593 bp upstream *sahH*, Gm^r-gfp fragment from pPS858 *Sac*I digested, and 473 bp downstream of the coding region of *sahH* between *KpnI* and *XbaI*	(Thoma, 2009)
pST09	Ap^r Gm^r; pEX18Ap with upstream *metF* and downstream of the coding region of *metF* between *KpnI* and *XbaI*, Gm^r-gfp fragment from pPS858 *Sac*I digested	(Thoma, 2009)
pST10	pUC20T containing a 872 bp *Sac*I- *Hind*III fragment of *metF* (PA0430), Carb^r, pUC20T::*metF*	(Thoma, 2009)
pMR01	Ap^r Gm^r; pEX18Ap with 751 bp upstream *metZ*, Gm^r-gfp fragment from pPS858 *Bam*HI digested, and 524 bp downstream of the coding region of *metZ* between *Kpn*I and *Xba*I	This study, Matthias. Rottmann(Rottmann, 2011)(Rottmann, 2011)(Rottmann, 2011)(Rottmann, 2011)(Rottmann, 2011)(Rottmann, 2011)
pAW03	Ap^r Gm^r; pEX18Ap with 752 bp upstream *mtnP*, Gm^r-gfp fragment from pPS858 *Bam*HI digested, and 522 bp downstream of the coding region of *mtnP* between *KpnI* and *XbaI*	This study
pFH04	pUC20T containing a 1048 bp *EcoR*I- *Xba*I fragment of *metF* (PA0430) and a 3840 bp *Xba*I *Hind*III fragment of *metH* (PA1843), Carb^r, pUC20T::*metF*::*metH*	This study, Frederike Haack
pFH06	Ap^r Gm^r; pEX18Ap with 809 bp upstream *metE*, Gm^r-gfp fragment from pPS858 *Bam*HI digested, and 613 bp downstream of the coding region of *metE* between *Sac*I and *Xba*I	This study, Frederike Haack
pFH07	Ap^r Gm^r; pEX18Ap with 460 bp upstream *metH*, Gm^r-gfp fragment from pPS858 *Bam*HI digested, and 837 bp downstream of the coding region of *metH* between *Sac*I and *Xba*I	This study, Frederike Haack
pSB1075	Ap^r, *ColE1 ori*, fusion of *lasRI::luxCDABE*, pUC18 plasmid backbone	(Winson *et al.*, 1998)
pSB406	Ap^r, *ColE1 ori*, fusion of *rhlRI::luxCDABE*, pUC18 plasmid backbone	(Winson *et al.*, 1998)

Table 22: Primer (restriction sites are underlined)

Primer	Primer sequence (5' - 3')	Restriction site	Construct
pST09 *metF*:			
metFP1	GGGGTACCGCCGATCACTACAACCAG	*Kpn*I	upstream fragment
metFP2	GAGCTCCGCGCCTCTTCTTGTTGATT	*Sac*I	upstream fragment
metFP3	GAGCTCTGGCGATCTGGAAGAATCTC	*Sac*I	downstream fragment
metFP4	GCTCTAGACGATGACGCCGGAGTTGA	*Xba*I	downstream fragment
pST06 *sahH*:			
sahHP1	GGAGTACCACCAGGGCGCTGCACATC	*Kpn*I	upstream fragment
sahHP2	CGAGCTCTGACAGCGCTCATGCGTAA	*Sac*I	upstream fragment
sahHP3	CGAGCTCCGGACACCTATCGCTACTA	*Sac*I	downstream fragment
sahHP4	GCTCTAGATGGCTGCAGGTTGGTGAT	*Xba*I	downstream fragment
pMR01 *metZ*:			
oAW208	GGGGTACCTCGTTGTCGGCAAGCACTA	*Kpn*I	upstream fragment
oAW207	CGGGATCCGCCATCCTCAGCATGAGTT	*Bam*HI	upstream fragment
oAW152	CGGGATCCGCCGCTCTCCCCTTTGTCA	*Bam*HI	downstream fragment
oAW153	GCTCTAGACCAGGCGGCGCGGAAGAAA	*Xba*I	downstream fragment
pAW03 *mtnP*:			
oAW154	GGGGTACCGCCTATGCGCTTGTTCCGT	*Kpn*I	upstream fragment
oAW155	CGGGATCCGGCGTTGCACGATGGCATT	*Bam*HI	upstream fragment
oAW162	CGGGATCCCTCCCTCAATCAATCAG	*Bam*HI	downstream fragment
oAW163	GCTCTAGAAGCATTTTCCCGGTCAC	*Xba*I	downstream fragment
pFH06 *metE*:			
oFH01	CCGAGCTCGCGGGCTACTCGGAAATCTA	*Sac*I	upstream fragment
oFH02	CGGATCCCGTGGGTGTTCCTCCAGTCG	*Bam*HI	upstream fragment
oFH03	CGGGATCCCGGGGCCTGGTGAGCGGAA	*Bam*HI	downstream fragment
oFH04	GCTCTAGAGCCATCGCGACGCGGACTT	*Xba*I	downstream fragment
pFH07 *metH*:			
oFH05	CCGAGCTCGCGCTGTATCGGCAGAAGTA	*Sac*I	upstream fragment
oFH06	CGGGATCCCGATGGACCTGGCGTCAGT	*Bam*HI	upstream fragment
oFH07	CGGGATCCCGCGAACCTTGGCTACGAT	*Bam*HI	downstream fragment
oFH08	CGTCTAGAGCCAGGGCATCAAGAACCT	*Xba*I	downstream fragment
pST10:			
forward	CGAGCTCAGCACTACCGCCAGTACTA	*Sac*I	pUC20T::*metF*
reverse	CCCAAGCTTTCGTGGATCGCGTGAGA	*Hind*III	pUC20T::*metF*
pFH04 *metF*::*metH*:			
oFH13	GGAATTCCGAGCACTACCGCCAGTACT	*EcoRI*	Forward primer *metF*
oFH15	GCTCTAGAGCGATGCTTGCGTGAGTTG	*XbaI*	Reverse primer *metF*
oFH17	GCTCTAGACGAAGCCCCGGCCTTTGTT	*XbaI*	Forward primer *metH*
oFH18	CCCAAGCTTGGGCTTGCGCGGCTTTTC	*Hind III*	Reverse primer *metH*
pAW08:			
oAW194	GGAATTCCATATGATGGTCGCGTCCAA	*NdeI*	pET-14b::*metF*
oAW195	CCGCTCGAGTCAGCGTGGCAGCTGGAG	*XhoI*	pET-14b::*metF*

Supplemental Material:

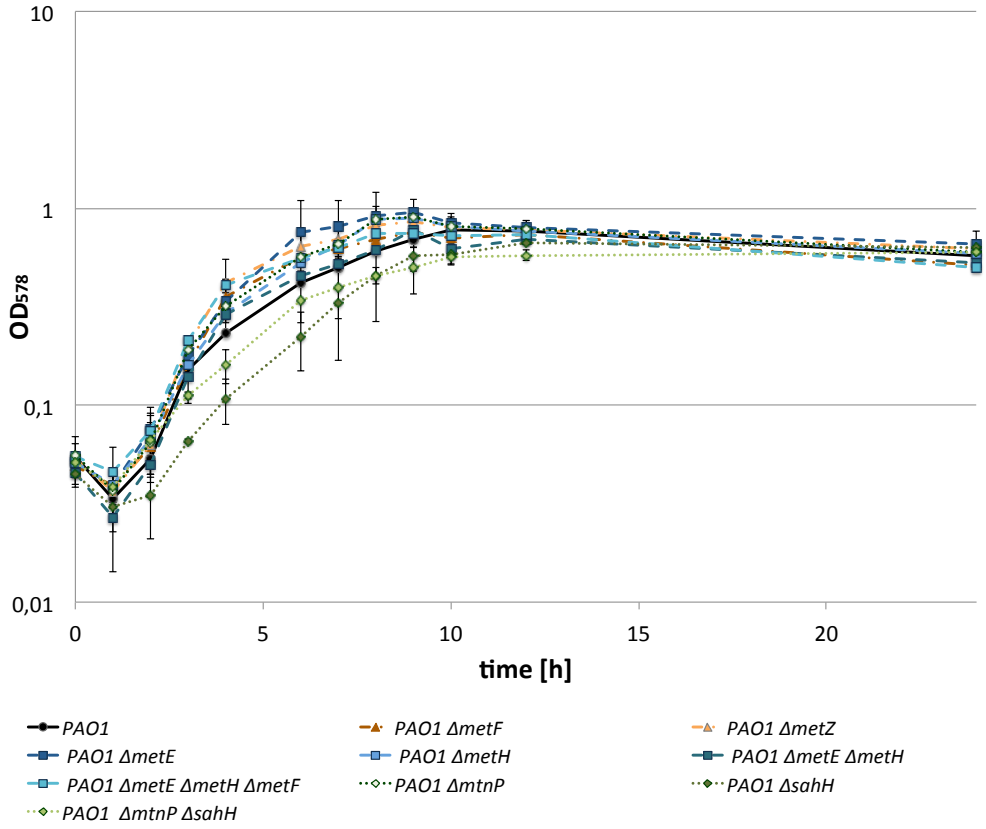


Fig. 24: Growth of methionine metabolism *P. aeruginosa* PAO1 deletion mutants in M9 20 mM succinate with 50 µM methionine supplementation

3.5 Acknowledgements

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4 Ribavirin against *Pseudomonas aeruginosa*

4.1 Introduction

The need for new antibiotic substances and new antibiotic targets is undeniable especially in the time of rising bacterial antibiotic resistance. Antibiotics target life processes such as cell-wall biosynthesis and cell envelope, protein biosynthesis and ribosomes, RNA and DNA replication and the folate metabolism (Walsh, 2003).

Multidrug resistant opportunistic pathogens such as the Gram-negative bacterium *Pseudomonas aeruginosa,* become more and more threating human pathogens, as it possesses intrinsic drug resistance (Hancock, 1998; Stover *et al.*, 2000). *P. aeruginosa* is known to cause nosocomial infections especially in immunocompromised patients. Acute and persistent infections are frequently caused by *P. aeruginosa* in the lungs of cystic fibrosis (CF) patients.

CF is a hereditary disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Hauser *et al.*, 2011; Lyczak *et al.*, 2002).

Once a chronic *P. aeruginosa* infection is established, the infection is nearly impossible to cure and often associated with the decline of lung function (Hauser *et al.*, 2011). But, how does *P. aeruginosa* achieve this state of resistance? Besides its intrinsic resistance, further changes of phenotype are observed, as *P. aeruginosa* colonizes the nutrient rich mucus in the lungs of CF patients. This special environment leads to the known phenotypes of mucoidy and the formation of microcolony and biofilm (Boucher *et al.*, 1997; Govan and Deretic, 1996; Lam *et al.*, 1980). Bacteria prevent phagocytosis and the diffusion of antibiotics is hindered (Lewis, 2001; Xu *et al.*, 2000). Nutrient availability also has a direct influence on bacterial metabolism and thereby influences growth and virulence factor production (Palmer *et al.*, 2005). Oxygen limitation in the microaerobic to anaerobic environment of the CF lung further reduces antibiotic effectiveness and also leads to phenotype changes (Alvarez-Ortega and Harwood, 2007; Worlitzsch *et al.*, 2002).

Since resistance against commonly used CF antibiotics such as tobramycin and ciprofloxacin is increasing, new effective substances and new targets are necessary (Amini *et al.*, 2011; Brazas *et al.*, 2007; Wu *et al.*, 1999). This study reports about both, a new antibiotic target and about an already known viral drug effective against *P. aeruginosa*.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is as a purine analogue. It is structurally very similar to adenosine (Ado), e.g. inter alia the product of the hydrolysis of S-adenosylhomocysteine (SAH) into homocysteine (Hcy) and Ado (Fig. 25A.&B.). Ribavirin is an approved drug against DNA and RNA viruses, e.g. for hepatitis C treatment in combination with interferon (Davis *et al.*, 1998). Recently ribavirin's impact on *Trypanosmoma cruzi´s* (Tc) S-adenosylhomocysteine hydrolase SAHH was successfully tested (Cai *et al.*, 2010; Cai *et al.*, 2007). Ribavirin resulted in total inhibition of the enzyme in an *in vitro* assay. Kruszewska *et al.* (2002) tested ribavirin successfully in their study looking for antimicrobial activity in non-antibiotic drugs. In addition Kruszewska *et al.* could show an effect against *P. aeruginosa*.

The potential drug target S-adenosylhomocysteine hydrolase (SahH, EC 3.3.1.1) has been identified in one of the essential metabolic pathways the activated methyl circle (AMC), exactly

in the recycling of S-adenosylmethionine (SAM) (Fig. 25). SAM is the second most frequently used enzyme substrate after ATP (Fontecave *et al.*, 2004; Loenen, 2006). The majority of estimated 95 % of SAM is used for methylation reactions (Griffith, 1987). Methylation is one of the most ubiquitous chemical reactions in cellular metabolism (Stepkowski *et al.*, 2005). The product of these reactions SAH is a potent inhibitor of transmethylation reactions. SAH is effectively converted to Hcy and Ado by SahH. SahH is a very important enzyme in the metabolism since methylation of macromolecules and small molecules is dependent on the SAM / SAH equilibrium.

Recently the intracellular SAM and SAH level for the *Escherichia coli* wild type (wt) strain MG1665 (OD_{600} of 1.62 ± 0.16) was identified with a ratio of 300:1 with 0.4 mM and 1.3 µM, respectively (Halliday *et al.*, 2010). In contrast to *P. aeruginosa*, *E. coli* and several other bacteria use a two-step mechanism instead, to convert SAH to Hcy. First S-ribosyl-homocysteine (SRH) and adenine are generated by Pfs a 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase. In a second step SRH is converted by LuxS a S-ribosylhomocysteine lyase to Hcy and 4,5-dihydroxy-2,3-pentanedione (DPD), which spontaneously forms autoinducer-2 a quorum sensing molecule (Halliday *et al.*, 2010; Parveen and Cornell, 2011; Winzer *et al.*, 2002).

Approximately 2-5 % of cellular SAM is used for decarboxylation reactions e.g. for the production of polyamines, quorum sensing molecules synthesis such as N-acylhomoserine lactones (AHLs) or as source of 5´deoxyadenosyl radicals. 5´deoxyadenosyl radicals are used for the production of vitamins e.g. biotin and lipoate. (Griffith, 1987; Parveen and Cornell, 2011). These reactions yield 5´-methylthioadenosine (MTA) or 5´deoxyadenosine (5´dAdo), respectively.

Sun *et al.* investigated 138 bacterial species. 60 of the investigated species contained a SAH hydrolase, whereas 51 possessed MTA/SAH nucleosidase. Few bacteria carry both enzymes, while symbionts and intracellular pathogens lack both and are therefore probably dependent on their hosts (Sun *et al.*, 2004; Winzer *et al.*, 2002).

P. aeruginosa is one of the few bacteria, which possess a SAH hydrolase as well as a MTA phosphorylase (MtnP, EC 2.4.2.28) for recycling of SAH and MTA, and probably for 5´dAdo (Challand *et al.*, 2010; Challand *et al.*, 2009; Choi-Rhee and Cronan, 2005). The narrow substrate specificity of both enzymes for the recycling of either SAH or MTA seems to be an advantage for survival of *P. aeruginosa* under the varying environmental conditions (Stepkowski *et al.*, 2005). This makes SahH an excellent drug target.

So far SahH is described as a homo tetrameric enzyme (Tanaka *et al.*, 2004). It contains tightly bound NAD^+ as cofactor. The structures of human, rat, mouse and of the malaria parasite *Plasmodium falciparum* SAHH are available (Ishihara *et al.*, 2010; Tanaka *et al.*, 2004; Turner *et al.*, 2000; Yamada *et al.*, 2005). The crystal structures for the bacterial SahHs of *Mycobacterium tuberculosis* and *Corynebacterium glutamicum* were determined (Lozada-Ramirez *et al.*, 2008; Reddy *et al.*, 2008). Structure dependent drug design is possible for these SAH hydrolases.

Other regulating factors seem to have an important impact on AMC genes and might be potentially targeted by drugs. Riboswitches are mRNA structures that regulate gene expression

(Blount and Breaker, 2006). For several bacterial species SAM and SAH dependent riboswitches were shown or were predicted in the mRNAs of the genes involved in the AMC. For *Pseudomonas syringae* a SAH dependent riboswitch in the mRNA of the *sahH* operon was described (Wang *et al.*, 2008). Wang *et al.* proposed that SAH binding activates gene expression. 15 SAH RNA motifs were identified in the Pseudomonadales (Wang *et al.*, 2008). Recently, a SAM-III box was identified in the 5´untranslated region (5´ UTR) of *metK* in *Enterococcus faecalis* (Smith *et al.*, 2010).

So far it is predicted and partly verified for some organisms that the genes of S-adenosylhomocysteine hydrolase (*sahH*), cobalamin-dependent methionine synthase (*metH*) and methylenetetrahydrofolate reductase (*metF*) are possessing a possible SAH element in the 5´ UTR (Wang *et al.*, 2008; Weinberg *et al.*, 2007).

Here, the possibility of ribavirin usage against *P. aeruginosa* PAO1 was examined. SahH as potential drug target in *P. aeruginosa* and the mode of action of ribavirin was investigated. The influence of SahH and MtnP on bacterial phenotype was examined. The presence of SahH in minimal medium, under simulated respiratory conditions and in CF sputum samples was determined. The impact of ribavirin on *P. aeruginosa* PAO1 was determined under simulated respiratory tract conditions. In addition, the presence of riboswitches in the 5´ UTR of AMC genes in *P. aeruginosa* was investigated. SahH was recombinantly produced and the impact of the inhibitor ribavirin on enzyme level was analysed *in vitro*.

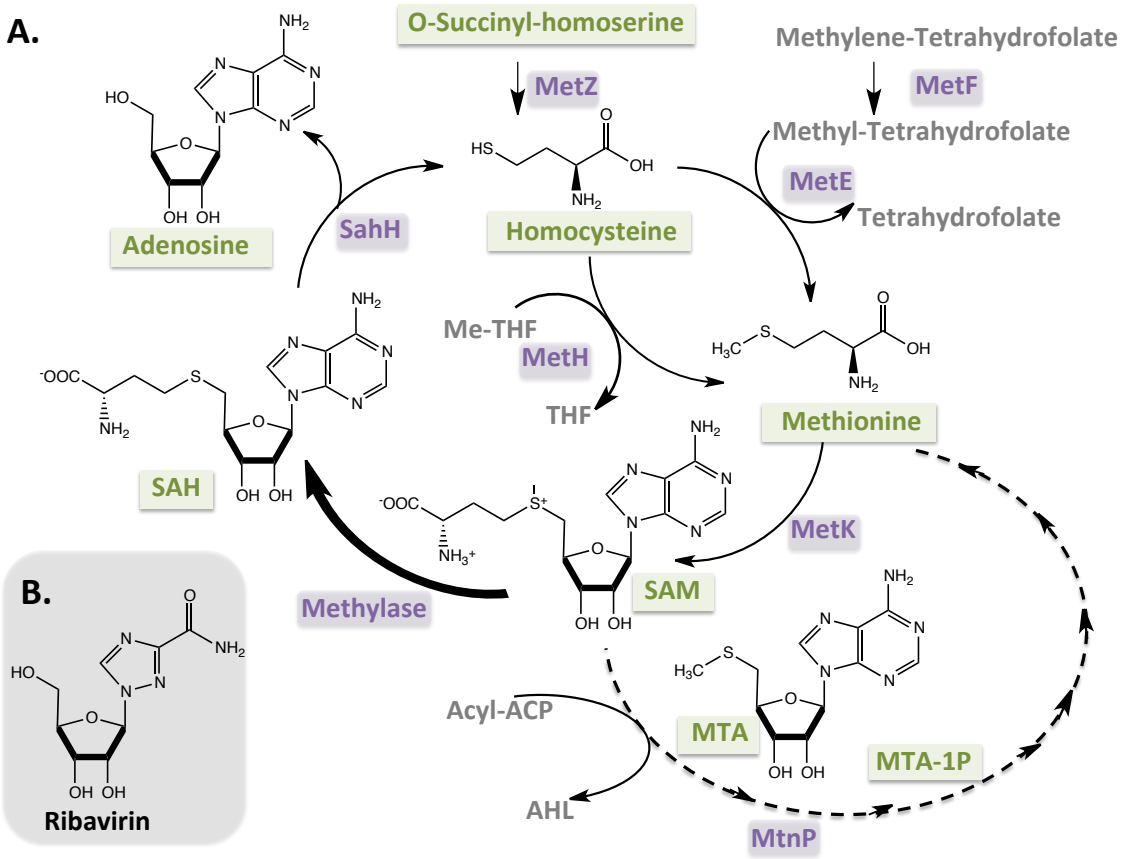

Fig. 25: Activated methylcycle (AMC) and SAM recycling in *Pseudomonas aeruginosa*

(A) Methionine and ATP are converted by S-adenosylmethionine synthetase MetK into SAM, phosphate and diphosphate. SAM is used for various metabolic reactions in the cell. If SAM is used for methylation the remaining SAH is hydrolysed by SahH into Ado and Hcy. Hcy can be generated out of O-succinyl-homoserine by MetZ. Hcy can be converted by either cobalamin-independent methionine synthase MetE or cobalamin-dependent methionine synthase MetH back into methionine. Both enzymes use the cofactor methyl-tetrahydrofolate (Me-THF) as methyl group donor. If SAM is used for other reactions e.g. by AHL synthase or as source of an amino propyl group for the generation of spermidine these reactions yield MTA. MTA is recycled by 5´-methylthioadenosine phosphorylase MtnP and by several other enzymes to methionine. Adapted from (Fontecave *et al.*, 2004; Heurlier *et al.*, 2006; Parveen and Cornell, 2011; Sekowska *et al.*, 2004)

(B) Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is as purine analogue structurally very similar to Ado.

4.2 Results & Discussion

4.2.1 High ribavirin concentrations lead to decreased growth in *Pseudomonas aeruginosa* PAO1.

Kruszewska *et al.* (2002) described the antimicrobial activity of ribavirin against *P. aeruginosa.* To analyse if the antimicrobial activity of ribavirin has also a partial effect on *P. aeruginosa* PAO1 growth, the growth of PAO1 in the presence of different ribavirin concentrations was investigated (Fig. 26). Concentrations of 1 - 10 µg/ml ribavirin did not affect *P. aeruginosa* PAO1 growth. 50 µg/ml ribavirin led to decreased growth of PAO1 up to 7 h. The incubation with 100 µg/ml resulted in even further decrease of growth. Only half of the optical density at 578 nm (OD_{578}) of the wild type (wt) was reached after 11 h. 250 µg/ml and 500 µg/ml led to growth inhibition and even reduction of OD_{578} in the latter case.

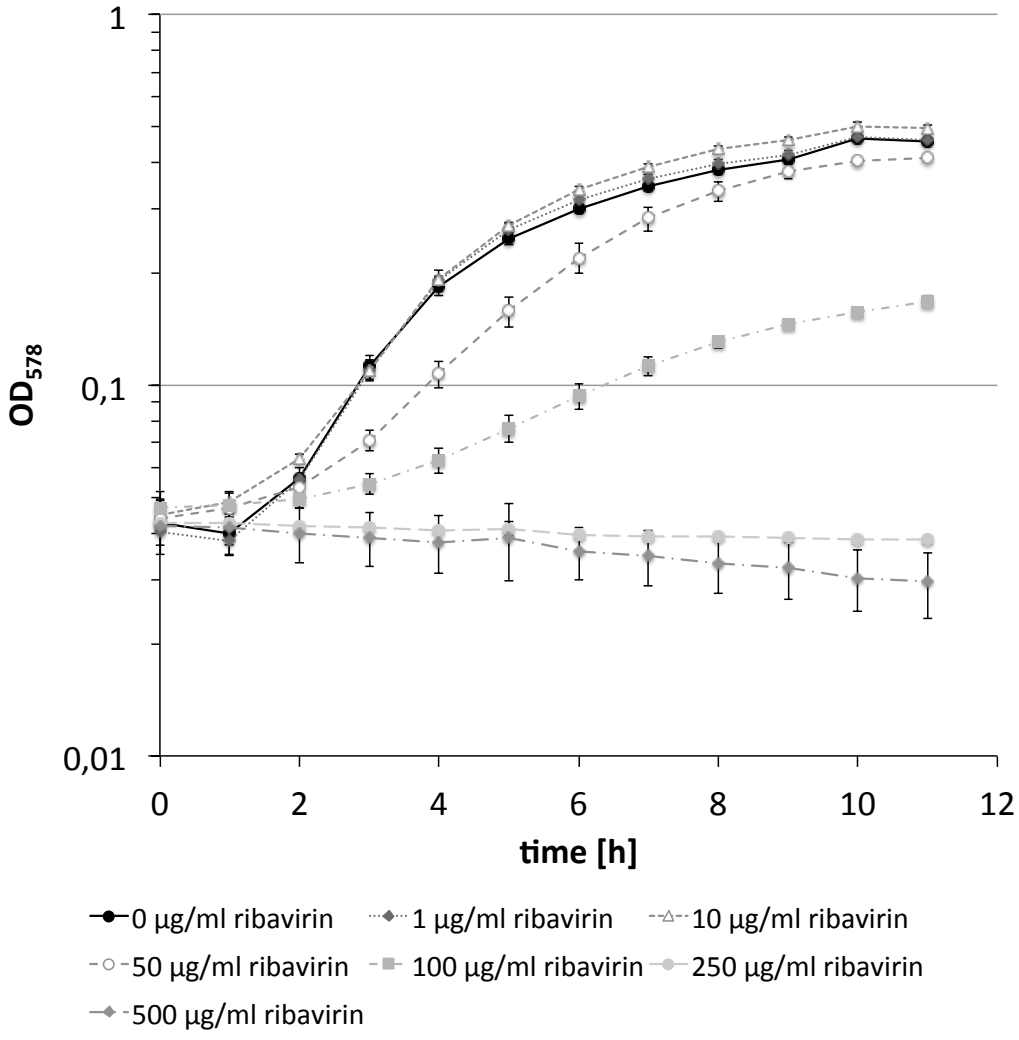

Fig. 26: Growth of *P. aeruginosa* PAO1 in the presence of different ribavirin concentrations

P. aeruginosa PAO1 was grown in M9 succinate 50 µM methionine supplementation with varying ribavirin concentrations while OD_{578} was measured. PAO1 control, grown without ribavirin (black circle, solid line), grew like PAO1 1 µg/ml ribavirin (grey diamond, dashed line) and PAO1 10 µg/ml ribavirin (grey triangle, dashed line). Supplementation of 50 µg/ml ribavirin (open circle, dashed line) led to decreased growth of PAO1 during the first 7 h. 100 µg/ml ribavirin (grey square, dashed

line) further decreased growth. 250 µg/ml ribavirin (grey circle, dashed line) and PAO1 500 µg/ml ribavirin (light grey diamond, dashed line) led to growth inhibition.

Ribavirin showed effects on *P. aeruginosa* PAO1 growth in concentrations ≥ 50 µg/ml. Drastic growth effects were observed for ribavirin concentrations > 100 µg/ml. Since Cai *et al.* (2007) described the successful inhibition of *T. cruzi* SAHH by ribavirin, we constructed a PAO1 Δ*sahH* deletion mutant. In order to investigate, if the deletion of *sahH* led to similar growth as it can be observed for PAO1 wt with ribavirin supplementation (Fig. 26). This would be the case, if *sahH* was the main target of ribavirin in the cell. And if SahH is a target, MtnP could also be a possible target, since both enzymes are involved in the recycling of either SAH or MTA respectively as part of AMC. Therefore, a PAO1 Δ*mtnP* deletion mutant was constructed and investigated.

An effect of ribavirin on transcription and translation was observed by Gupta *et al.* (2012) in mononuclear blood cells of HCV patients treated with Peg-interferon α and ribavirin. A similar effect of ribavirin on *P. aeruginosa* transcription and translation was also observed in our study especially on the AMC genes.

4.2.2 *Pseudomonas aeruginosa* PAO1 Δ*sahH* shows drastically decreased growth.

The first step to analyse the influence of the genes *sahH* (PA0432) and *mtnP* (PA3004) was to investigate the growth phenotypes of *P. aeruginosa* PAO1 Δ*sahH*, PAO1 Δ*mtnP* and PAO1 Δ*sahH* Δ*mtnP*. The methionine metabolism mutant PAO1 Δ*metZ* was examined.

The majority of SAM is used for methylation reactions of various molecules (Griffith, 1987) and therefore yields SAH. But since SAH is probably recycled by SahH in *P. aeruginosa*, an influence on phenotype in a PAO1 Δ*sahH* strain was expected.

P. aeruginosa PAO1 wt and AMC metabolism deletion strains were cultivated in M9 minimal medium, with 50 µM methionine supplementation, respectively. PAO1 wt showed no difference when grown in the presence or absence of methionine (Fig. 27). But PAO1 Δ*sahH* showed decreased growth even in the presence of methionine compared to wt. Nearly no growth at all was observed when grown without methionine supplementation (Fig. 27A), which was also detected for the methionine auxotrophic PAO1 Δ*metZ* strain (Fig. 27D). But in contrast to PAO1 Δ*sahH,* PAO1 Δ*metZ* showed wt like growth when supplemented with methionine. The growth defect of PAO1Δ*sahH* was similar to the growth defect of PAO1 wt in the presence of 50 µg/ml ribavirin (Fig. 27A), when both strains were grown with 50 µM methionine supplementation.

No growth phenotype was observed for PAO1 Δ*mtnP* (Fig. 27C) in M9 with or without methionine, respectively. A PAO1 Δ*sahH* Δ*mtnP* mutant strain showed the same growth phenotype as the PAO1 Δ*sahH*. The *mtnP* deletion seems not to increase the impact.

The decreased growth of PAO1 Δ*sahH* showed clearly the importance of SahH as recycling enzyme for SAH into Hcy and Ado. Growth, though decreased, of PAO1 Δ*sahH* was still possible in the presence of methionine. Methionine taken up, was possibly able to replenish the methionine pool and thereby refilling the SAM pool of the cell. Since methionine formation out of Hcy might be impaired in a PAO1 Δ*sahH* mutant strain and as Hcy level might be low whereas the SAH level might be high. Furthermore, the cellular SAM level might be low. Probably the

high SAH level were leading to inhibition of methylase reactions. All this might have contributed to the decreased growth observed for PAO1 Δ*sahH* in the presence of methionine. Without external methionine, the SAM pool might be to low, and the SAH pool to high to allow methylations, which are necessary for growth.

The deletion of *mtnP* showed no such effects. This might be because only a minor part of SAM is recycled via MtnP. In addition no further decrease in growth was visible in a PAO1 Δ*sahH* Δ*mtnP* double deletion strain. It was assumed that MtnP is not capable of recycling SAH instead of MTA. The enzymes seem to have a narrow substrate specificity as supposed by Stepkowski *et al*. (2005). Furthermore, it was concluded that the deletion of SahH leads to methionine auxotrophy, as previously described for *Rhodobacter capsulatus* (Sganga *et al.*, 1992), or very impaired methionine synthesis.

Since SAH hydrolases are very important enzymes in bacteria and influence growth drastically, it is definitely a potential drug target also in *P. aeruginosa.* Since inhibition of SahH should result in the same decreased growth or growth arrest as revealed by the deletion. Moreover, 50 µg/ml ribavirin led to a similar growth phenotype of PAO1 wt as the deletion *sahH* caused. This observation indicates that SahH might be a possible target of ribavirin in the cell. Moreover, it was confirmed that MtnP is not targeted by ribavirin, since the deletion of *mtnP* resulted in no growth effect.

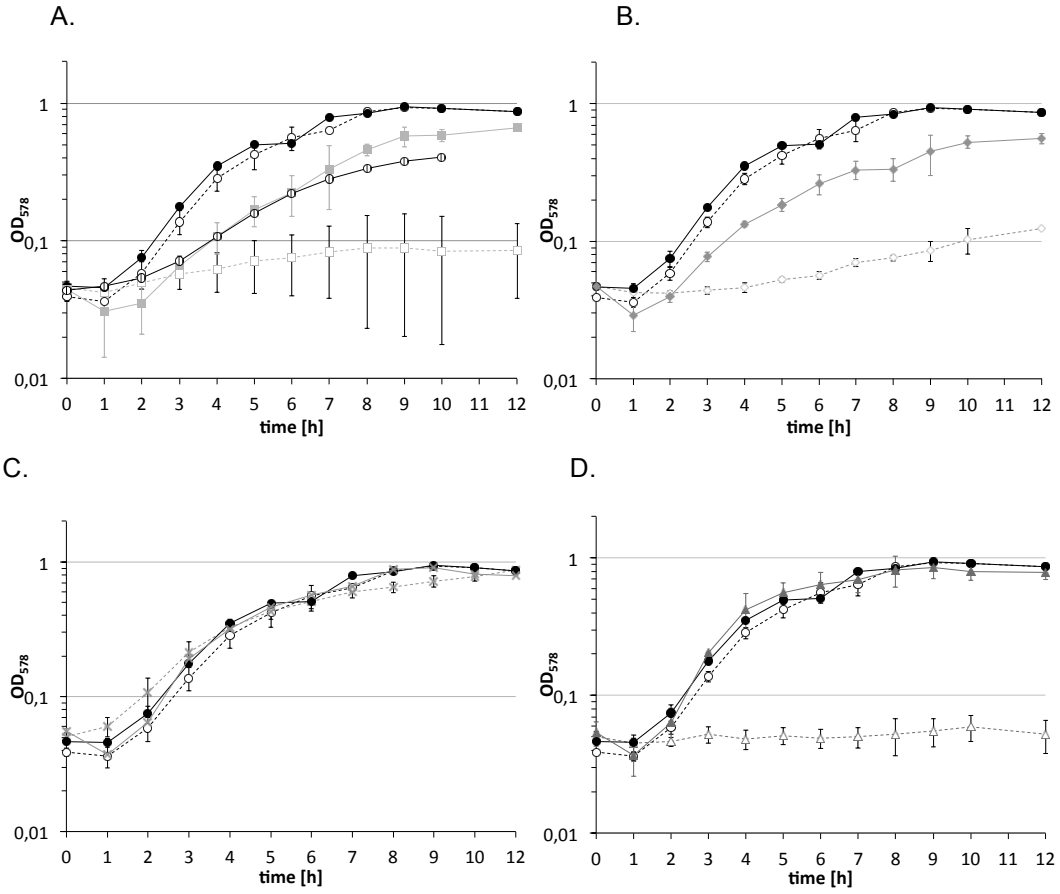

Fig. 27: Growth curves of *P. aeruginosa* PAO1 wt, PAO1 Δ*sahH*, PAO1 Δ*sahH* Δ*mtnP*, PAO1 Δ*mtnP* and PAO1 Δ*metZ* in M9 succinate

P. aeruginosa PAO1 and mutant strains were grown in M9 succinate with (closed symbols, solid line) and without (open symbols, dashed line) 50 µM methionine supplementation. Furthermore,

PAO1 wt was grown in the presence of 50 µg/ml ribavirin with methionine supplementation (open symbol with black strip, solid line)

(A) PAO1 was grown with 50 µM methionine (black circle, solid line), without methionine (open circle, dashed line) and in the presence of 50 µg/ml ribavirin with methionine supplementation (open symbol with black strip, solid line). PAO1 Δ*sahH* was grown with 50µM methionine (grey square) and without methionine (open square, dashed line). No difference in growth in correlation with methionine was visible for the wt. In contrast, nearly no growth was observed for PAO1 Δ*sahH* without methionine supplementation. The critical growth conditions are also reflected by the high standard derivation for this condition. Even in the presence of methionine, growth of PAO1 Δ*sahH* was impaired compared to the wild type (wt) till stationary phase is reached. A similar growth pattern as for PAO1 ∆*sahH* can be observed for PAO1 wt incubated with 50 µg/ml ribavirin, both in the presence of 50 µM methionine.

(B) PAO1 was grown with 50 µM methionine (black circle, solid line) and without methionine (open circle, dashed line) and PAO1 Δ*sahH* Δ*mtnP* was grown with 50 µM methionine (grey diamond) and without methionine (open diamond, dashed line). The same growth pattern as for PAO1 ∆*sahH* was observed, there is no further addition due to deletion of *mtnP*.

(C) PAO1 was grown with 50 µM methionine (black circle, solid line) and without methionine (open circle, dashed line) and PAO1 Δ*mtnP* was grown with 50 µM methionine (grey x) and without methionine (grey x, dashed line). PAO1 ∆*mtnP* showed wt like growth behaviour.

(D) PAO1 was grown with 50 µM methionine (black circle, solid line) and without methionine (open circle, dashed line) and PAO1 Δ*metZ* was grown with 50 µM methionine (grey triangle) and without methionine (open triangle, dashed line). PAO1 Δ*metZ* showed wt like growth behaviour when grown in the presence of methionine and no growth without methionine supplementation.

Experiments were performed in dublicates. The standard deviation is shown.

4.2.3 SahH is detectable in *Pseudomonas aeruginosa* culture samples and also present in high amounts under simulated respiratory tract conditions.

In order to analyse if the SahH protein is detectable in CF sputum samples and in *P. aeruginosa* culture, Western blot analysis using a polyclonal anti-*P. aeruginosa* (Pa) SahH antibody purified from rabbit serum was used. The SahH was detected in *P. aeruginosa* PAO1 culture after 5 h of aerobic cultivation in M9 minimal medium (Fig. 28B). With a high specificity only SahH was detected and not the recombinant *P. aeruginosa* MtnP (Guan *et al.*, 2011), which is also involved in SAM recycling by converting MTA as MTA nucleosidase (Parveen and Cornell, 2011). Both enzymes share 10 % identity on amino acid level.

SahH seems to be an important factor under simulated respiratory tract conditions. In the proteome of *P. aeruginosa* PAO1 high amounts of SahH were identified by mass spectrometry, when grown as biofilm on ASM under anaerobic conditions (Thoma, 2009).

But no SahH was detectable in Western blot analysis of CF-sputum samples. Though all samples contained high amounts of protein as is visible in the SDS-PAGE (Fig. 28C), since the detection is very sensitive, neither bacterial SahH was detected, nor cross detection of human SAHH took place. This might be because the CF patients were not colonised at the point of sampling or the samples did not contain sufficient amounts of *P. aeruginosa*. No CFU control was possible since samples were frozen at -80°C and no additional analysis was performed.

To further verify that only Pa SahH was detected, blood samples were analysed since human SAHH can be isolated from human serum and erythrocytes (Arredondo-Vega *et al.*, 1989; Hershfield *et al.*, 1979). As expected no human SAHH was detected in the erythrocyte, serum or monocyte fraction (Fig. 28D), even though the percent identity of Pa SahH and *Homo sapiens* (Hs) SAHH is 57 % (Fig. 29).

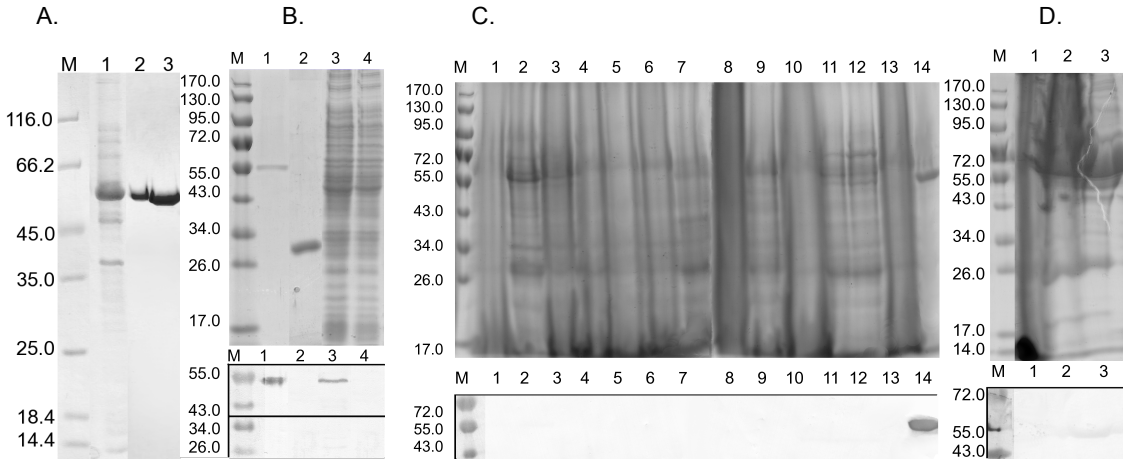

Fig. 28: Production and purification of recombinant SahH from *P. aeruginosa* (Pa) and detection of Pa SahH in CF-sputum samples and specificity testing of anti-Pa SahH antibody against SAHH in human blood sample

(A) SDS-PAGE analysis of the production and purification of SahH. Lane M, marker proteins with M_r indicated; lane 1, proteins within cell-free extract of *E. coli* BL21 (DE3) carrying pET14b::*sahH* after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG); lane 2, recombinant His-SahH after chromatography on Ni Sepharose™ 6 Fast Flow ; lane 3, SahH after thrombin cleavage.

(B) SDS-PAGE analysis and Western blot of *P. aeruginosa* PAO1 and PAO1 Δ*sahH* culture after growth of 5 h in M9 succinate. Lane M, marker proteins with M_r indicated; lane 1, recombinant Pa His-SahH; lane 2, recombinant Pa His-MtnP; lane 3, proteins of PAO1 culture; lane 4, proteins of PAO1 Δ*sahH* culture.

Western blot analysis of the SahH production in the supernatant of PAO1 and PAO1 Δ*sahH* was detected by anti-Pa SahH antibody.

(C) SDS-PAGE analysis of 13 different CF-sputum samples. Lane M, marker proteins with M_r indicated; lane 1, proteins of CF-sputum sample 132; lane 2, proteins of CF-sputum sample 177; lane 3, proteins of CF-sputum sample 025; lane 4, proteins of CF-sputum sample 154; lane 5, proteins of CF-sputum sample 090; lane 6, proteins of CF-sputum sample 153; lane 7, proteins of CF-sputum sample 369; lane 8, proteins of CF-sputum sample 373; lane 9, proteins of CF-sputum sample 091; lane 10, proteins of CF-sputum sample 089; lane 11, proteins of CF-sputum sample 372; lane 12, proteins of CF-sputum sample 368; lane 13, proteins of CF-sputum sample 148; lane 14, recombinant Pa His-SahH.

Underneath a Western blot of the SDS-PAGE is shown using a polyclonal anti-Pa SahH rabbit antibody and an alkaline phosphatase conjugated goat anti-rabbit secondary antibody. Lane 1-13, CF-sputum samples; Lane 14, recombinant Pa His-SahH. SahH was not detected in the CF-sputum samples.

(D) SDS-PAGE and Western blot analysis of human blood samples fractions. The specificity of the SahH antibody was tested against different blood components and detected by anti-Pa SahH

antibody. Lane M, marker proteins with M_r indicated; lane 1, proteins of erythrocyte fraction; lane 2, proteins of the serum faction; lane 3, proteins of monocyte fraction.

4.2.4 SAH hydrolases are a highly conserved among bacteria and eukaryotes.

The alignment of amino acid sequences of different SAH hydrolases performed by ClustalW2 was used to determine the percent identity (Larkin *et al.*, 2007). Several SAH hydrolases already crystallised or investigated as potential drug targets were compared (Fig. 29). The sequences of Hs SAHH, *Rattus norvegicus* (Rn) SAHH, *Trypanosaoma cruzi* (Tc) SAHH, *Mycobacterium tuberculosis* (Mt) SahH, *Corynebacterium glutamicum* (Cg) SahH, *Plasmodium falciparum* (Pf) SAHH and Pa SahH were used (Cai *et al.*, 2010; Cai *et al.*, 2007; Ishihara *et al.*, 2010; Lozada-Ramirez *et al.*, 2008; Reddy *et al.*, 2008; Tanaka *et al.*, 2004; Turner *et al.*, 2000; Yamada *et al.*, 2005) .

The highest level of percent identity was found for the eukaryotic hydrolases Hs SAHH with Rn SAHH with 96 %. The second highest percentage of identity was detected for Hs SAHH with Tc SAHH with 73 %. Furthermore Mt SahH and Cg SahH showed the highest percent of identity for bacterial SahHs with 75 %. The percent identity of Pa SahH aligned with the other hydrolases ranged from 58 % with Rn SAHH and 57 % with Hs SAHH to 50 % with Cg SahH. The 12 amino acids between AA 270 - 282 in the Pa SahH were not found in other here investigated hydrolases and for AA 353 – 360 applies the same (Fig. 29). Stepkowski *et al.* (2005) obtained similar results in their alignment of bacterial SahHs. They also showed that only *Synechococcus* sp. PCC 7002 contains the same 12 amino acids (Pa SahH AA 270 – 282) in its sequence. This clearly shows that SAH hydrolases are highly conserved enzymes and very important for the hydrolysis of SAH into Hcy and Ado. 57 % percent identity on amino acid level of Hs SAHH and Pa SahH shows a high degree of conservation, but leaves also amino acid differences. Thereby potentially leading to structural differences, catalytical differences and differences in substrate specificity. Cai *et al.* (2007) showed that even though the SAHH sequences of Hs and Tc are highly similar with 73% percent identity, the inactivation of Tc SaHH by the nucleoside analogue ribavirin is 5 fold faster than the inactivation of the human enzyme.

```
H.sapiens           --------------MSDKLPYKVADIGLAAWGRKALDIAENEMPGLMRMRERYSASKPLK 46
R.norvegicus        --------------MADKLPYKVADIGLAAWGRKALDIAENEMPGLMRMREMYSASKPLK 46
T.cruzi             --------------MTD---YKVRDISLAEWGRKAIEIAENEMPGLMELRREYSQSKPLK 43
M.tuberculosis      MTGNLVTKNSLTPDVRNGIDFKIADLSLADFGRKELRIAEHEMPGLMSLRREYAEVQPLK 60
C.glutamicum        ------------------MDFKVADLSLAEAGRHQIRLAEYEMPGLMQLRKEFADEQPLK 42
P.falciparum        --------------MVENKSKVKDISLAPFGKMQMEISENEMPGLMRIREEYGKDQPLK 45
P.aeruginosa        --------MSAVMTPAGFTDYKVADITLAAWGRRELIIAESEMPALMGLRRKYAGQQPLK 52
                                      *: *: **  *:  : ::* ***.** :*. :.  :***

H.sapiens           GARIAGCLHMTVETAVLIETLVTLGAEVQWSSCNIFSTQDHAAAAIAKA---------GI 97
R.norvegicus        GARIAGCLHMTVETAVLIETLVALGAEVRWSSCNIFSTQDHAAAAIAKA---------GI 97
T.cruzi             GAKIAGCLHMTVQTAVLIETLIQLGAEVRWSSCNIFSTQDNAAAAIAKR---------GI 94
M.tuberculosis      GARISGSLHMTVQTAVLIETLTALGAEVRWASCNIFSTQDHAAAAVVVGPHGTPDEPKGV 120
C.glutamicum        GARIAGSIHMTVQTAVLIETLTALGAEVRWASCNIFSTQDEAAAAIVVG-SGTVEEPAGV 101
P.falciparum        NAKITGCLHMTVECALLIETLQKLGAQIRWCSCNIYSTADYAAAAVSTL--------ENV 97
P.aeruginosa        GAKILGCIHMTIQTGVLIETLVALGAEVRWSSCNIFSTQDQAAAAIAAA---------GI 103
                    .*:* *.:***:: .:*****  ***:::*.****:** * ****:           .:
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H.sapiens           PVYAWKGETDEEYLWCIEQTLY-FK--DGPLNMILDDGGDLTNLIHT------------- 141
R.norvegicus        PVFAWKGETDEEYLWCIEQTLH-FK--DGPLNMILDDGGDLTNLIHT------------- 141
T.cruzi             PVFAWKGETEEEYQWCIEQTLKGFSG-DGFPNMILDDGGDLTNHVLD------------- 140
M.tuberculosis      PVFAWKGETLEEYWWAAEQMLTWPD-PDKPANMILDDGGDATMLVLRGMQYEKAG----V 175
C.glutamicum        PVFAWKGESLEEYWWCINQIFSWG---DELPNMILDDGGDATMAVIRGREYEQAG----L 154
P.falciparum        TVFAWKNETLEEYWWCVESALTWGDGDDNGPDMIVDDGGDATLLVHKGVEYEKLYEEKNI 157
P.aeruginosa        PVFAWKGETEEEYEWCIEQTILKDG-QPWDANMVLDDGGDLTEILHK------------- 149
                    .*:***.*: *** *. :. :          :*::***** *  :

H.sapiens           ----------------------------KYPQLLPGIRGISEETTTGVHNLYKMMANGIL 173
R.norvegicus        ----------------------------KHPQLLSGIRGISEETTTGVHNLYKMMANGIL 173
T.cruzi             ----------------------------HCPHLVDKIYGISEETTTGVKNLYKRLQRGKL 172
M.tuberculosis      VPPAEEDDPAEWKVFLNLLRTRFETDKDKWTKIAESVKGVTEETTTGVLRLYQFAAAGDL 235
C.glutamicum        VPPAEANDSDEYIAFLGMLREVLAAEPGKWGKIAEAVKGVTEETTTGVHRLYHFAEEGVL 214
P.falciparum        LPDPEKAKNEEERCFLTLLKNSILKNPKKWTNIAKKIIGVSEETTTGVLRLKKMDKQNEL 217
P.aeruginosa        ----------------------------KYPQMLERIHGITEETTTGVHRLLDMLKNGTL 181
                                                :  ::   : *::******* .* .    . *

H.sapiens           KVPAINVNDSVTKSKFDNLYGCRESLIDGIKRATDVMIAGKVAVVAGYGDVGKGCAQALR 233
R.norvegicus        KVPAINVNDSVTKSKFDNLYGCRESLIDGIKRATDVMIAGKVAVVAGYGDVGKGCAQALR 233
T.cruzi             PIPAINVNDSVTKSKFDNLYGCRESLVDGIKRATDVMIAGKTACVCGYGDVGKGCAAALR 232
M.tuberculosis      AFPAINVNDSVTKSKFDNKYGTRHSLIDGINRGTDALIGGKKVLICGYGDVGKGCAEAMK 295
C.glutamicum        PFPAMNVNDAVTKSKFDNKYGTRHSLIDGINRATDMLMGGKNVLVCGYGDVGKGCAEAFD 274
P.falciparum        LFTAINVNDAVTKQKYDNVYGCRHSLPDGLMRATDFLISGKIVVICGYGDVGKGCASSMK 277
P.aeruginosa        KVPAINVNDSVTKSKNDNKYGCRHSLNDAIKRGTDHLLSGKQALVIGYGDVGKGSSQSLR 241
                     ..*:****:***.* ** ** *.** *.: *.** ::.** . : ********.: ::

H.sapiens           GFGARVIITEIDPINALQAAMEGYEVTT-------------MDEACQ-EGNIFVTTTGCI 279
R.norvegicus        GFGARVIITEIDPINALQAAMEGYEVTT-------------MDEACK-EGNIFVTTTGCV 279
T.cruzi             AFGARVVVTEVDPINALQAAMEGYQVLL-------------VEDIVE-QAHIFVTTTGND 278
M.tuberculosis      GQGARVSVTEIDPINALQAMMEGFDVVT-------------VEEAIG-DADIVVTATGNK 341
C.glutamicum        GQGARVKVTEADPINALQALMDGYSVVT-------------VDEAIE-DADIVITATGNK 320
P.falciparum        GLGARVYITEIDPICAIQAVMEGFNVVT-------------LDEIVD-KGDFFITCTGNV 323
P.aeruginosa        QEGMIVKVAEVDPICAMQACMDGFEVVS**PYKNGINDGTEAS**IDAALLGKIDLIVTTTGNV 301
                      *  * ::* *** *:** *:*:.*               ::     . .:.:* **

H.sapiens           DIILGRHFEQMKDDAIVCNIGHFDVEIDVKW-LNENAVEKVNIKPQVDRYRLK------- 331
R.norvegicus        DIILGRHFEQMKDDAIVCNIGHFDVEIDVKW-LNENAVEKVNIKPQVDRYLLK------- 331
T.cruzi             DIITAEHFPRMQDDAIVCNIGHFDTEIQVSW-LKANAKERVEVKPQVDRYTMH------- 330
M.tuberculosis      DIIMLEHIKAMKDHAILGNIGHFDNEIDMAG-LERSGATRVNVKPQVDLWTFGD------ 394
C.glutamicum        DIISFEQMLKMKDHALLGNIGHFDNEIDMHSLLHRDDVTRTTIKPQVDEFTFS------- 373
P.falciparum        DVIKLEHLLKMKNNAVVGNIGHFDDEIQVNELFNYKGIHIENVKPQVDRITLP------- 376
P.aeruginosa        NVCDANMLKALKKRAVVCNIGHFDNEIDTAF--MRKNWAWEEVKPQVHKIHRT**GKDGFDA** 359
                    ::   . :  ::. *:: ****** **:       .      :****.

H.sapiens           -NGRRIILLAEGRLVNLGCAMGHPSFVMSNSFTNQVMAQIELWTHPD--KYPVG------ 382
R.norvegicus        -NGHRIILLAEGRLVNLGCAMGHPSFVMSNSFTNQVMAQIELWTHPD--KYPVG------ 382
T.cruzi             -NGRHIILLAEGRLVNLGCASGHPSFVMSNSFSNQVLAQIELWTQRESGKYPRGEKA--Q 387
M.tuberculosis      -TGRSIIVLSEGRLLNLGNATGHPSFVMSNSFANQTIAQIELWTKNDE--YDNE------ 445
C.glutamicum        -TGRSIIVLSEGRLLNLGNATGHPSFVMSNSFADQTIAQIELFQNEGQ--YENE------ 424
P.falciparum        -NGNKIIVLARGRLLNLGCATGHPAFVMSFSFCNQTFAQLDLWQNKDTNKYENK------ 429
P.aeruginosa        **H**NDDYLILLAEGRLVNLGNATGHPSRIMDGSFANQVLAQIHLFEQKYADLPAAEKAKRLS 419
                     ..  :*:*:.***:*** * ***: :*. ** :*.:**:.*: :

H.sapiens           VHFLPKKLDEAVAEAHLGKLNVKLTKLTEKQAQYLGMSCDGPFKPDHYRY 432
R.norvegicus        VHFLPKKLDEAVAEAHLGKLNVKLTKLTEKQAQYLGMPINGPFKPDHYRY 432
T.cruzi             VYFLPKKLDEKVAALHLGKLGAKLTKLTARQADYINCPVDGPFKPDHYRY 437
M.tuberculosis      VYRLPKHLDEKVARIHVEALGGHLTKLTKEQAEYLGVDVEGPYKPDHYRY 495
C.glutamicum        VYRLPKVLDEKVARIHVEALGGQLTELTKEQAEYIGVDVAGPFKPEHYRY 474
P.falciparum        VYLLPKHLDEKVALYHLKKLNASLTELDDNQCQFLGVNKSGPFKSNEYRY 479
P.aeruginosa        VEVLPKKLDEEVALEMVKGFGGVVTQLTPKQAEYIGVSVEGPFKPDTYRY 469
                    *  *** *** **   :  :.  :*:*  .*.:::.    **:*.: ***
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Fig. 29: Alignment of bacterial and eukaryotic SAH hydrolases with Pa SahH

Amino acid sequence alignment of *H. sapiens* (Hs) SAHH (432 AA), *R. norvegicus* (Rn) SAHH (432 AA), *T. cruzi* (Tc) SAHH (437 AA), *M. tuberculosis* (Mt) SahH (495AA), *C. glutamicum* (Cg) SahH (474 AA), *P. falciparum* (Pf) SAHH (479 AA) and *P. aeruginosa* (Pa) SahH (469 AA) using ClustalW2 (Larkin *et al.*, 2007). The percent identity of Pa SahH and Hs SAHH is 57 %, Pa SahH and Rn SAHH is 58 %, of Pa SahH and Tc SAHH is 56 %, of Pa SahH and Mt SahH is 51 %, of Pa SahH and Cg SahH is 50 % and of Pa SahH and Pf SAHH is 54 %, respectively. The UniProtKB accession numbers of the hydrolases are for Hs SAHH (P23526), Rn SAHH (P10760), Tc SAHH (Q7YUF0) Mt SahH (A5U7S2), Cg SahH (Q8NSC4), Pf SAHH (P50250) and Pa SahH (Q9I685) as indicated. Amino acids only present in the Pa sequence in this alignment are marked in bold letters.

4.2.5 Deletion of *sahH* leads to impaired growth under simulated respiratory tract conditions.

As already mentioned SahH is present under simulated CF respiratory tract conditions in high amounts (Thoma, 2009). Furthermore, PAO1 Δ*sahH* also showed a decreased growth rate in biofilms on polyvinyl filter on ASM (Sriramulu *et al.*, 2005) under anaerobic conditions CF compared to the wt even with sufficient amounts of methionine (Thoma, 2009), similar to growth behaviour in M9 with methionine supplementation.

4.2.6 Inhibition of SahH under simulated respiratory tract conditions by the nucleoside analogue ribavirin.

Since SahH was also very important under simulated CF conditions, the potential already approved drug ribavirin was tested. Ribavirin is as a purine analogue structurally very similar to Ado, the product of the hydrolysis of SAH into Hcy and Ado (Fig. 25A & B). Ribavirin licensed against DNA and RNA viruses e.g. for hepatitis C treatment in combination with interferon (Davis *et al.*, 1998).

Treatment of stationary phase biofilm with 2 mg/ml ribavirin for 12 h (Thoma, 2009) led to a drastically reduced survival rate of *P. aeruginosa* PAO1 wt with only 30 % survival in the biofilm model under simulated respiratory tract conditions. Ribavirin seems to be a promising drug to decrease *P. aeruginosa* PAO1 survival even in biofilms under simulated CF conditions.

4.2.7 Does slow growth of PAO1 Δ*sahH* lead to altered antibiotic resistance?

Some might argue that the impaired growth leads to altered antibiotic resistance in PAO1 Δ*sahH*. Slow growth often results in an altered resistance, since commonly used antibiotics target life processes and if metabolic processes, e.g. cell wall biosynthesis in the case of β-lactams, are slowed down the antibiotics are not as effective. Two studies using transposon libraries found for the PA14 *sahH* (PA14_05620) transposon mutant and the PAO1 *sahH* (PA0432) orthologous transposon mutant altered antibiotic resistance. One study using Vitek 2 for semiautomatic susceptibility testing observed an altered resistance against the fluoroquinolone levofloxacin of 3 fold change in the MIC (in Log_2) compared to the Pa14 wt. Whereas the growth rate was determined with 0.45 compared with 1.0 for the wt (Dotsch *et al.*, 2009). Moreover, they remarked that no mutant exhibited serious growth alterations. No

alteration for tobramycin resistance was detected. But on the other hand the study performed by Schurek *et al.* (2007) classified the *sahH* gene in the class of tobramycin resistance genes. PA14 *sahH* (PA14_05620) transposon mutant was described as general growth defect mutant causing defective growth in LB broth (Schurek *et al.*, 2008).

Independently from the results, these high-throughput studies identified *sahH* as an important factor. But most antibiotic resistance studies are performed under laboratory conditions, which do not simulate the conditions in the natural environment or the host. This leads to different results concerning MIC and resistance in general. Therefore, a closer look on transcription and translation in PAO1 Δ*sahH* was taken to exclude that only growth is the factor for altered resistance or effectiveness of drugs.

4.2.8 Differences in transcription and translation of AMC genes in PAO1 Δ*sahH* compared to PAO1 wt.

The influence of a *sahH* deletion on transcription and translation was investigated using transcriptional and translational *lacZ* reporter gene fusions. The regulation of AMC genes was of especial interest. Therefore, bacteria were grown as described in Material and Methods. Bacteria were harvested when reaching a similar OD_{578} of 0.300 ± 0.05. That was the case after 4 h for PAO1 and after 6 h for PAO1 Δ*sahH.*

Interestingly, the transcription of *sahH* operon and *metK* operon was 5.5-fold and 3.7-fold induced compared to the PAO1 wt, respectively. This progress was observed for translation with 9.8-fold and 10.3-fold for the *sahH* operon and the *metK* operon, respectively (Table 23 and Fig. 30). This means that the probably high SAH level, which was already determined on molecule level for *E. coli pfs* and *luxS* mutants (Halliday *et al.*, 2010), probably influences the transcription of both operons positively. Furthermore, the translation of *metK* was even further increased. This strongly supports the assumption of possible ribozyme regulation in the 5´UTR of *metK* and *sahH*. The high level of SAH or the low level of SAM might lead to expression of *sahH* operon and *metK* operon, respectively. Direct ligand binding might lead to a stabilisation of secondary and tertiary structures, and thereby might make the Shine-Dalgarno (SD) sequence accessible for the ribosome and the initiation of translation possible (Wang *et al.*, 2008).

The transcription level of the two methionine synthases genes of *P. aeruginosa metH* and *metE* was 1.3-fold and 1.8-fold induced (Table 23). MetH is a cobalamin dependent and the encoded MetE is a cobalamin independent methionine synthase. 3.6-fold induced is *metH* translation but in contrast is *metE* translation - 14.0-fold decreased compared to PAO1 wt. The PAO1 Δ*sahH* mutant seems to use MetH to a higher degree, as the expression of *metH* is clearly upregulated. This might be because of a potential SAH riboswitch in the 5´UTR of the *metH* operon. Binding of SAH or absence of SAM might make the SD sequence accessible for translation.

The presence of SAH or SAM dependent riboswitches in the 5´UTR of *sahH*, *metK* and *metH* seems possible from the results of β-galactosidase assays.

Table 23: Fold change of transcription and translation in PAO1 Δ*sahH* compared to the samples of PAO1 wt [MU / MU]

	PAO1 Δ*sahH*			
	Transcriptional fusion (mini-CTX-*lacZ*)		Translational fusion (pME6013)	
P_{sahH}	5.5	± 0.1	9.8	± 0.1
P_{metK}	3.7	± 0.3	10.3	± 0.2
P_{metH}	1.3	± 0.1	3.6	± 0.1
P_{metE}	1.8	± 0.1	-14.0	± 0.8

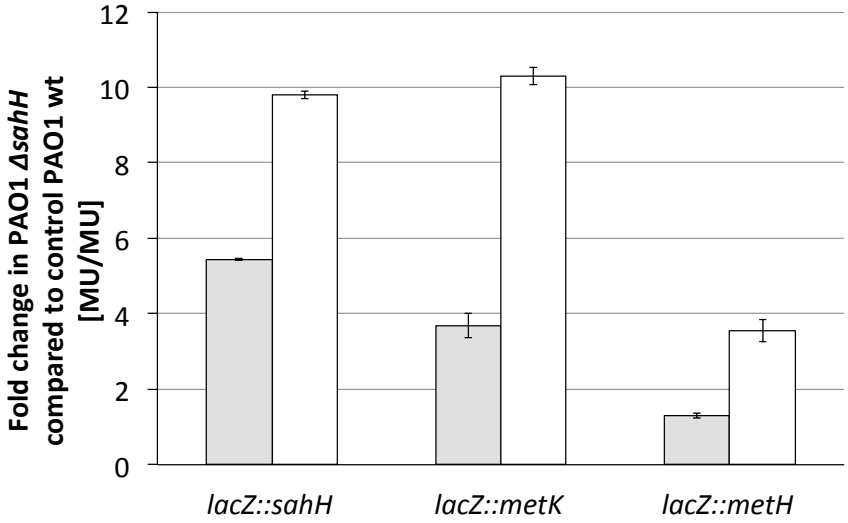


Fig. 30: Influence of the deletion of *sahH* on transcription and translation in PAO1 Δ*sahH* compared to PAO1 wt

Fold change of transcription (mini-CTX-*lacZ,* grey bars) and translation (pME6013, white bars) of the genes *sahH, metK* and *metH* was determined in PAO1 Δ*sahH*. The fold change was compared to PAO1 wt.

The standard deviation of two representative experiments is shown. The value of the empty control vectors mini-CTX-*lacZ* and pME6013, performed for each condition tested, was subtracted.

4.2.9 A potential SAH riboswitch in the 5´untranslated region of *sahH* gene.

For the 5´untranslated region (5´UTR) of *sahH* gene of *P. syringae* a SAH riboswitch was described (Wang *et al.*, 2008). Therefore, an alignment of 150 bp upstream of start codon of *sahH* gene of *P. syringae pv. phaseolicola* 1448A (PSPPH_0451), *P. syringae pv. tomato str.* DC3000 (PSPTO_5068) and *P. aeruginosa* PAO1 (PA0432) was performed. The percentage of sequence identity was 94 % for *P. syringae pv. phaseolicola* 1448A and *P. syringae pv. tomato str.* DC3000 (Fig. 31A). Furthermore, revealed the comparison of PAO1 *sahH* sequence to *P. syringae sahH* a high level of conservation of bases and base pairing in the stem loops proposed for *P. syringae sahH* by Wang *et al.* (2008). The proposed loop regions of *P. syringae* do not contain the poly U in *P. aeruginosa*. This can also be observed in the high percentage of identity in the alignment of PAO1 sahH of 71 % for *P. syringae pv. phaseolicola* 1448A and 67 % for *P. syringae pv. tomato str.* DC3000 (Fig. 31A). The sequence alignment is showing the

high level of conservation and therefore makes the presence of a SAH dependent riboswitch in the 5´UTR very likely.

A.

```
PSPPH_0451 P. syringae      3´GUA-ACUCUCAGAGGUAAGGUAUUUUUACGCUUACCCGCGGCAAC-ACGC 48
PSPTO_5068 P. syringae      3´GUA-ACUCUCAGAGGUAAGGUAUUUUUACGCUUACCCGCGGCAAC-ACGC 48
PA0432     P. aeruginosa    3´GUACGCAUUGAGAGGUAAGCAACA--GACGCUUACCCGCGGCAACCACGC 48
                             ***  *  * *********  *     ****************** ****

PSPPH_0451 P. syringae       AAAUUCCAAACGGCCAGCUCCUAGCAGGACCGUUUGUUGCGGGGUAGGCU 98
PSPTO_5068 P. syringae       AAAUUCCGAACGGCCGACAC-UAGUUGGACUGUUUGUUGCGGGGUAGGCU 97
PA0432     P. aeruginosa     AACUGUC---UGGCCG-CUC-CGGCCGGACU-CUCGC-GCGAGGCAAGCU 91
                             ** *  *    ****  * *   *  ****   * *  *** ** * ***

PSPPH_0451 P. syringae       CGGACUGUCCC-UUUUGGACGACGUCGCGGGGAGCCUGUCCACCGCCCUU 147
PSPTO_5068 P. syringae       CGGACUGUCCC-AUUUGGACGACGUCGCGGGGAGCCUGUCCACCGCCCUU 146
PA0432     P. aeruginosa     CGGACUGUCCGGAUCCGGACGACGUCGCGGGGAGCUUGCCUUCCGCCCUC 141
                             **********   *  ******************** ** *  *******

PSPPH_0451 P. syringae       GCCAUA------ 5´ 153
PSPTO_5068 P. syringae       GCCAUAG----- 5´ 153
PA0432     P. aeruginosa     GCUGGGCUUCCC 5´ 153
                             **
```

B.

```
metK P. aeruginosa      3´GUAGUUAGCCAAGGAUGUAAUGGCCACCCACUCCGC----CGAAA-GCCUACGU-----A 50
metH P. aeruginosa      3´GUACCUA-CCUGGACCGCAGUCACCCGCCUUUCCACUUUUCGUAUUGUUUCCGUCCCCGA 59
sahH P.aeruginosa       3´GUACGCA--UUGAGAGGUAAGCAACAGACGCUUACC----CGCGGCAACCACGC-----A 49
                          ***   *         * *     *   *  *   *    **         **      *

metK P. aeruginosa       GGGACCG--CUUCAC-GGCCUGG-ACCUAGACCUUCGGUAACGC------AUUUGGUUAC 100
metH P. aeruginosa       AAGGCCGGCCCCCACCAGCCUGGUACUUAAA------GUAA---------GUUCAACUUC 104
sahH P. aeruginosa       ACUGUCUGGCCGCUCCGGCCGGACUCUCGCGCG--AGGCAAGCUCGGACUGUCCGGAUCC 107
                              *   *  * *  *** *   *           * **          *     * *

metK P. aeruginosa       AUUUCU----GAGAGCGGCCCGCACUC---GGGACGCCGCAGCCGGGUGACCCGGUCCAG 5´ 153
metH P. aeruginosa       CUACAA----GUAAGCGCCCGGCCUUUUUAGGGAC--CGCGGCAAAACAGC---GUCA-- 5´ 153
sahH P. aeruginosa       GGACGACGUCGCGGGGAGCUUGCCUUC-------CGCCCUCGCUGGGCUUC----CC--- 5´ 153
                                    *   *   *  **  *        *  *   **       *     *
```

Fig. 31: Alignment of potential riboswitches in the 5´UTR of the mRNA of sahH and AMC genes

(A) Alignment of 150 bp upstream of start codon of the *sahH* gene of *P. syringae pv. phaseolicola* 1448A (PSPPH_0451), *P. syringae pv. tomato str.* DC3000 (PSPTO_5068) and *P. aeruginosa* PAO1 (PA0432) using Clustal W. For *P. syringae* a SAH dependent riboswitch was predicted by Wang *et al.* (2008).

(B) Alignment of 150 bp upstream of start codon of *P. aeruginosa* PAO1 *metK*, *metH* and *sahH* gene. Start codon and SD-sequence are marked.

The percentage of identity of the upstream regions of *metK*, *metH* and *sahH* was compared because the β-galactosidase assays indicated the possibility of riboswitches activated by high SAH levels or low SAM levels. The percent identity is 50 % for the alignment of *sahH* and *metK* (Fig. 31B). For *sahH* and *metH* it is 41 % and for *metH* and *metK* it is 53 %. Since riboswitches share certain conservation on sequence level, but more importantly in secondary structure, no conclusion can be drawn from these results, because sequence identity is not highly conserved.

On the other hand, it is not clear, if the 150 bp upstream of *metK* and *metH* really contain the riboswitch, nor if the riboswitches all belong to the same class.

4.2.10 Downregulation of transcription and translation of AMC genes due to ribavirin.

Several other studies investigated the influence of potential SahH inhibitors on enzyme level. The influence on transcriptional and translational level using *lacZ* reporter gene fusions of the AMC genes was also investigated. PAO1 and PAO1 Δ*sahH* were grown in the presence of 50 µg/ml ribavirin, respectively. 50 µg/ml ribavirin led to growth of the PAO1 wt like PAO1 Δ*sahH* mutant in M9 supplemented with methionine (Fig. 27A).

It was assumed that if ribavirin leads to inhibition of *sahH* in PAO1 wt, the same pattern of transcription and translation must be observed, as it is seen in PAO1 Δ*sahH*, since transcription and translation seem to be strongly influenced by the elevated SAH level and lower SAM level. The fold change of the ribavirin treated PAO1 wt and PAO1 Δ*sahH* was compared to the untreated PAO1 wt or the untreated PAO1 Δ*sahH*, respectively.

But in contrast, it was observed that the fold change of transcription and translation was decreased for all investigated AMC genes in PAO1 treated with ribavirin (Table 24 and Fig. 32A). The strongest reduction in transcription was observed for *metH* in the wt with - 7.9-fold downregulation. The transcription of the genes *sahH* and *metK* was - 2.3-fold downregulated in the wt. Furthermore, the translation of *metK* and *sahH* was downregulated with - 3.5-fold and - 3.3-fold in wt, respectively. Downregulation of transcription and translation was also detected in PAO1 Δ*sahH.* The transcription of *metK* was with - 4.4-fold strongly downregulated in PAO1 Δ*sahH*. Moreover, the translation of *metH* was strongly decreased with - 8.9-fold in PAO1 Δ*sahH*. The translation of all other AMC genes in wt and PAO1 Δ*sahH* was approximately - 1.5 ± 0.3 -fold decreased.

Transcription and translation of the investigated AMC genes were always higher in PAO1 Δ*sahH* under all conditions (Table 25 and Fig. 32B). Only transcription of *metH* had the same level for wt and PAO1 Δ*sahH*. This higher level of transcription and translation can also be seen for the treatment with ribavirin for PAO1 Δ*sahH*. Here, was also *metH* the exception, but the translation was lower than in the wt even with ribavirin treatment (Fig. 32B). Translation of *sahH* was also very low in PAO1 (Fig. 32B). Furthermore, transcription and translation were always lower for the ribavirin treated strain compared to the untreated strain.

Table 24: Fold change of transcription and translation of AMC genes of PAO1 and of PAO1 Δ*sahH* in the presence of 50 µg/ml ribavirin compared to the untreated samples of PAO1 and PAO1 Δ*sahH* [MU / MU]

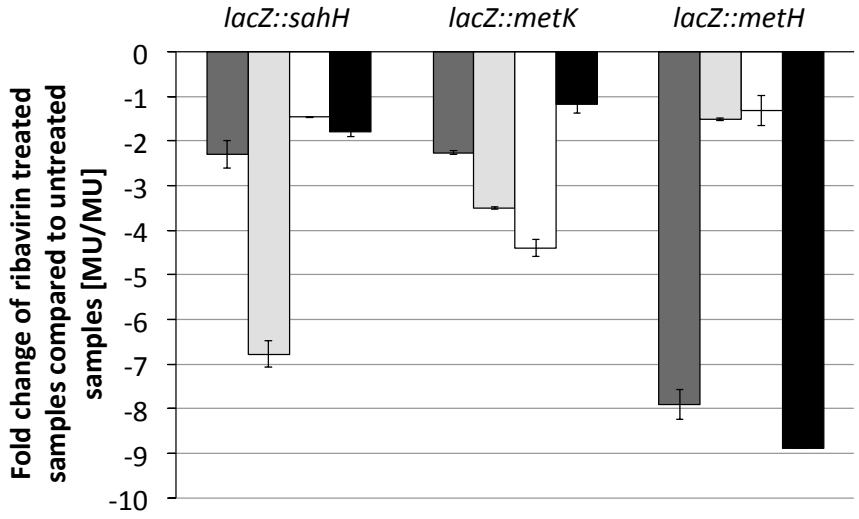
	Transcriptional fusion (mini-CTX-*lacZ*) with ribavirin				Translational fusion (pME6013) with ribavirin			
	PAO1		PAO1 Δ*sahH*		PAO1		PAO1 Δ*sahH*	
P_{sahH}	-2.3	± 0.3	-1.5	± 0.3	-6.2	± 0.2	-1.8	± 0.1
P_{metK}	-2.3	± 0.1	-4.4	± 0.1	-3.5	± 0.2	-1.2	± 0.2
P_{metH}	-7.9	± 0.3	-1.3	± 0.0	-1.5	± 0.3	-8.9	± 0.1

Table 25: Transcription and translation of AMC genes of PAO1 and of PAO1 Δ*sahH* in the presence and absence of 50 µg/ml ribavirin in Miller Units [MU]

	Miller Units [MU]	PAO1		PAO1 with ribavirin		PAO1 Δ*sahH*		PAO1 Δ*sahH* with ribavirin	
Transcription	mini-CTX-lacZ::sahH	3111	± 964	1353	± 949	16924	± 68	11560	± 153
	mini-CTX-lacZ::metK	5062	±1464	2232	± 207	18667	±1689	4242	±3456
	mini-CTX-lacZ::metH	916	± 553	115	± 304	1032	± 71	779	± 345
Translation	pME6013::sahH	4	± 3	0.6	± 0.5	23	± 17	13	± 2
	pME6013::metK	210	± 65	60	± 6	2167	± 54	1860	± 475
	pME6013::metH	337	± 145	222	± 10	1203	± 502	135	± 9

From these results was concluded that the major target of ribavirin in *P. aeruginosa* is not SahH. Ribavirin might have a more general effect on the bacterial cell. It might interact as a purine analogue in general with the transcription and translation machinery, as also the transcription and translation of the control vectors was reduced (data not shown). Suppression of ribosomal protein synthesis and protein translation factors was also observed in mononuclear blood cells of HCV patients treated with Peg-interferon α and ribavirin (Gupta *et al.*, 2012). Furthermore, the downregulation of transcription and translation of AMC genes was also detected in PAO1 Δ*sahH* (Table 24 and Fig. 32B).

A.



B.

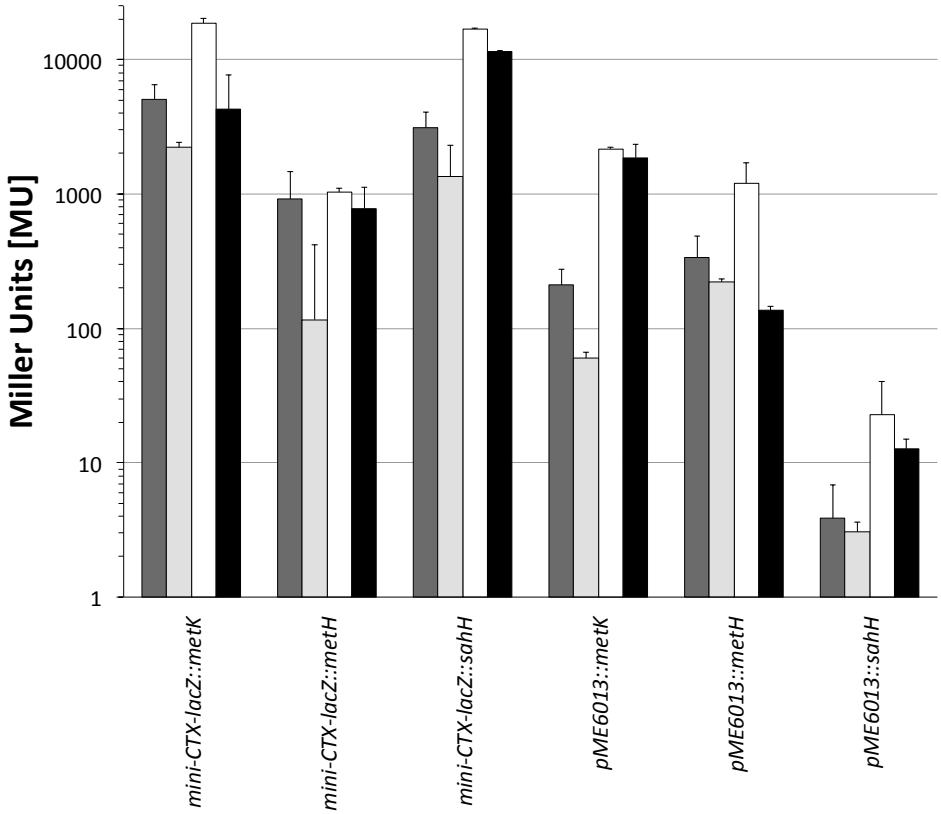


Fig. 32: Influence of 50 µg/ml ribavirin on transcription and translation in PAO1 wt and PAO1 Δ*sahH*

(A) Fold change of transcription (mini-CTX-*lacZ*) and translation (pME6013) of *sahH*, *metK*, and *metH* in PAO1 and PAO1 Δ*sahH* treated with 50 µg/ml ribavirin. The fold change is compared for PAO1 ribavirin to the untreated PAO1 wt and for PAO1 Δ*sahH* ribavirin to the untreated PAO1 Δ*sahH*. PAO1 fold change of transcription (grey bars), PAO1 fold change of translation (light grey bars) and PAO1 Δ*sahH* fold change of transcription (white bars) and PAO1 Δ*sahH* fold change of translation (black bars) are depicted.
The value of the empty control vectors mini-CTX-*lacZ* and pME6013, performed for each condition tested, was subtracted.
Experiments were performed independently three times. The standard deviation is indicated.

(B) β-Galactosidase activity in Miller Units [MU] of *sahH, metK* and *metH* in PAO1 and PAO1 Δ*sahH* treated with and without 50 µg/ml ribavirin. PAO1 (grey bars), PAO1 with 50 µg/ml ribavirin (light grey bars) and PAO1 Δ*sahH* (white bars) and PAO1 Δ*sahH* incubated with 50 µg/ml ribavirin (black bars) are exhibited, respectively.
The standard deviation of two representative experiments is shown. The value of the empty control vectors mini-CTX-*lacZ* and pME6013, performed for each condition tested, was subtracted.

4.2.11 Influence of ribavirin on SahH *in vitro*.

To investigate Pa SahH also on enzyme level, SahH was recombinantly produced and purified as N-terminal hexahistidine-tagged (His-tagged) protein with a theoretical molecular weight of

53.7 kDa (489 AA). The native SahH contains 469 amino acids (AA) and a theoretical molecular mass of 51.4 kDa (Fig. 28A).

To examine the effect of ribavirin *in vitro*, a SahH enzyme assay was applied to investigating the hydrolytic reaction. SAH is converted by SahH into Ado and Hcy. Therefore, SahH was incubated in a time-dependent manner as described previously by Cai *et al.* (2007) with and without 100 µM ribavirin. The rate of Hcy formation was connected with TNB^{2-} formation out of DNTB. Hcy and DNTB reacted to TNB^{2-}, which was detected at 412 nm. The reaction was driven by the deamination of Ado to inosine by the Ado deaminase.

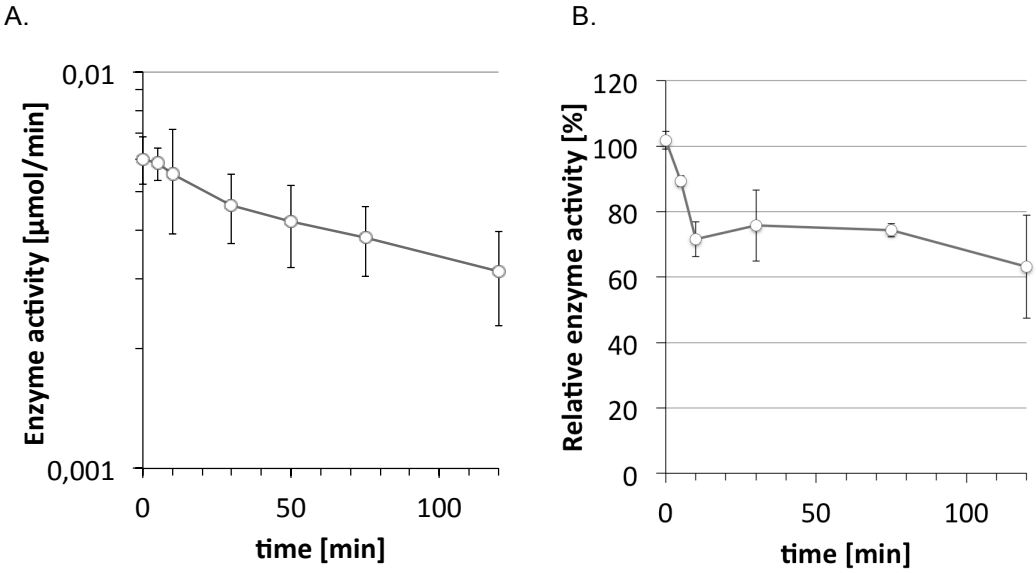


Fig. 33: Inhibition of SahH activity by 100 µM ribavirin time-dependent

Time-dependent loss of total and relative enzyme activity of Pa SahH. SahH was incubated with and without 100 µM ribavirin, respectively. The relative enzyme activity was calculated. The enzyme activity was measured in the hydrolytic direction.

(A) SahH was incubated time-dependent in phosphate buffer pH 7.2 and enzyme activity was measured in the presence of 100 µM ribavirin.

(B) Relative enzyme activity [%] of SahH incubated with 100 µM ribavirin was calculated by comparing SahH treated with 100 µM ribavirin with untreated SahH.

Experiments were performed independently two times. The standard deviation from the two representative experiments is shown.

Prolonged incubation of SahH with ribavirin up to 120 min led to continuous reduction of enzyme activity (Fig. 33A). The first reduction of relative enzyme activity to 72 % was relatively fast after only 10 min of incubation with ribavirin (Fig. 33B). After 120 min ribavirin resulted in 37 % less relative activity. The relative enzyme activity was reduced to 63 %.

For the Tc SAHH a higher level of inhibition was observed when incubated with 100 µM ribavirin after 120 min only 10 % activity remained. Whereas Hs SAHH still possessed around 70 % remaining activity (Cai *et al.*, 2007). Pa SahH seems to be very similar in the inhibition potential by ribavirin to Hs SAHH with still 63 % relative activity.

Perhaps other nucleoside analogues will inhibit Pa SahH more effectively than ribavirin. Nonetheless crystal structure of Pa SahH would be a very helpful tool to design new drugs that especially target the unique structures of Pa SahH.

4.3 Conclusions

This study confirms that ribavirin definitely shows antimicrobial activity against *P. aeruginosa* PAO1. High concentrations of ribavirin > 250 µg/ml during cultivation led to growth inhibition. Furthermore, incubation of mature *P. aeruginosa* PAO1 biofilm with 2 mg/ml ribavirin for 12 h under simulated respiratory tract conditions led to a survival rate of only 30 %. This means that ribavirin is effective under anaerobic CF conditions. Since the growth curves of PAO1 Δ*sahH* and PAO1 wt incubated with 50 µg/ml ribavirin were very similar and SahH was indicated as ribavirin target in *T. cruzi*, it was assumed that SahH could be a major target of ribavirin in *P. aeruginosa*.

No typical altered gene expression in PAO1 treated with ribavirin as in PAO1 Δ*sahH* was observed. In contrast, a totally unexpected downregulation of transcription and translation in both PAO1 and PAO1 Δ*sahH* treated with ribavirin was detected. Interestingly, ribavirin is very successful against PAO1 biofilm but the exact mode of action remains to be discovered.

This clearly shows that SahH is not the main target of ribavirin in *P. aeruginosa*. However, it was shown that the enzyme SahH is definitely a bottleneck enzyme in the essential part of bacterial metabolism and therefore a powerful drug target candidate. This is supported by the fact that a deletion of *sahH* has drastic effects on bacterial growth. Bacterial metabolism might be unbalanced because of the unbalanced intracellular SAH/SAM level. Thereby causing an altered transcription and translation of AMC genes and probably other genes. This different regulation seems to be an attempt of bacterial metabolism to antagonize the imbalances.

On the other hand, methionine auxotrophy was observed in *P. aeruginosa* PAO1 Δ*sahH* in methionine free medium. Since Hcy cannot be generated out of SAH by SahH in a PAO1 Δ*sahH* mutant. Therefore, the methionine pool cannot be refilled by the formation of methionine out of Hcy and Me-THF as cofactor by the enzymes MetE and MetH (Fig. 25A). This indicates, as previously described by Wang, *et al.* (2008), that the loss of Hcy pool is not compensated by increased *de novo* synthesis e.g. by MetZ pathway, but instead methionine is taken up from the medium to replenish the AMC. If the methionine pool is refilled, slow growth in a PAO1 Δ*sahH* mutant is still possible. Interestingly, a PAO1 Δ*sahH* Δ*mtnP* double mutant did not show an increased reduction of growth under the tested conditions. This indicates that there might still be other mechanisms of SAM and MTA recycling in *P. aeruginosa* to be discovered. SahH was detected by Western blot and proteome analysis in high amounts in minimal medium as well as in ASM under simulated respiratory tract conditions.

Sequence comparison revealed that the percent identity of human SAHH and Pa SahH is highly conserved with 57 %. But furthermore, two unique sequence areas were detected for Pa SahH. Moreover, sequence comparison of the 5´UTR region of *sahH* revealed a potential SAH dependent riboswitch, which seems to positively regulate transcription and translation of the *sahH* operon. This riboswitch might also be a potential target for newly designed drugs against *P. aeruginosa*.

The high inhibition rate of Tc SAHH by ribavirin could not be confirmed for Pa SahH. Pa SahH showed only 28 % reduced relative enzyme activity after short term incubation of 10 min and even long term incubation with 100 µM ribavirin only resulted in 63 % relative enzyme activity. This clearly shows that SahH is not the main target of ribavirin in *P. aeruginosa.*

The next step is to crystallize the Pa SahH to make the structural differences to the Hs SAHH obvious. Then Pa SahH structure dependent inhibitor design is possible in order to find new antibiotics against a powerful drug target. Ribavirin on the other hand is effective against *P. aeruginosa* PAO1 under CF conditions and as already approved drug ready for administration.

4.4 Material and Methods

4.4.1 Bacterial strains and growth conditions

All bacterial strains used in this study are shown Table 26 and plasmids in Table 27. *E. coli* and *P. aeruginosa* strains were grown routinely in LB medium for standard molecular biology protocols. For *P. aeruginosa* the following concentrations of antibiotics were used: carbenicillin 250 µg/ml, gentamicin 80 µg/ml, and tetracycline 100 µg/ml. For growth experiments ribavirin was added to the medium to achieve the concentrations of 1 µg/ml up to 500 µg/ml.

The *E. coli* strain DH10B was used as a host for cloning and *E. coli* strain BL21 (DE3) was used for protein production.

For growth experiments, *P. aeruginosa* was grown in M9 minimal medium (Sambrook and Russell, 2001) containing 1 x M9- salts [47.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl], 20 mM succinate, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 x trace metals [0.148 mg/l $ZnSO_4$ x 6 H_2O, 0.100 mg/l $MnCl_2$ x 4 H_2O, 0.236 mg/l $CoSO_4$ x 7 H_2O, 0.100 mg/l $NiCl_2$ x 6 H_2O, 0.020 mg/l $CuCl_2$ x 2 H_2O, 0.050 mg/l Na_2MoO_4 x 2 H_2O, 1 µl/l 25 % HCl modified after (Schlegel, 2007)] and 50 µM methionine, respectively. Cultures were inoculated at OD_{578} of 0.05 and shaken at 200 rpm at 37°C, if not indicated otherwise.

The results of at least two independent experiments are shown. Standard derivation is shown as error bars.

4.4.2 Construction of *Pseudomonas aeruginosa* deletion strains

To obtain unmarked gene deletion mutants the well-established strategies based on *sacB* counterselection and FLP recombinase excision (Hoang *et al.*, 1998) were used. To achieve deletion of *mtnP* (PA3004) the pAW03 suicide plasmid was constructed and in case of *metZ* (PA3107) pMR01.

For the construction of both plasmids the gentamycin resistance cassette of pPS858 was digested by *Bam*HI and was cloned between two PCR fragments of *mtnP* and *metZ* in the multiple cloning site of pEX18Ap, respectively. The two PCR fragments contained DNA homologous to upstream und downstream fragments of *mtnP* and *metZ* genes respectively, which were deleted in the respective mutant strains. Primer sequence and vector details are shown in Table 27 and Supplemental Table 28.

The construction of PAO1 Δ*sahH* is described elsewhere (Thoma, 2009). The gene deletion constructs were introduced into *E. coli* DH10B by transformation. Plasmids containing the correct insert were transferred via diparental mating using *E. coli* strain ST18 (Thoma and Schobert, 2009) in *P. aeruginosa* PAO1. After successful gene deletion the gentamicin resistance cassette was removed from the genome using the pFLP2 plasmid to obtain unmarked gene deletion mutants (Hoang *et al.*, 1998). The resulting mutants were verified by PCR.

4.4.3 Construction of transcriptional and translational promoter-*lacZ* reporter gene fusions

Chromosomal transcriptional promoter-*lacZ* reporter gene fusions were constructed using the mini-CTX-*lacZ* vector (Becher and Schweizer, 2000) and translational *lacZ* reporter gene

fusions were constructed using the pME6013 (Schnider-Keel *et al.*, 2000) to analyse the presence and activity of putative riboswitches (Wang *et al.*, 2008). To analyse the putative *sahH* (PA0432) promoter region, a 500 bp fragment upstream of the translational start and 159 bp of the coding region, a in total 659 bp PCR product, was amplified. Primer sequences and vector details are shown in Table 27 and Supplemental Table 28. The other constructs were generated likewise. Transfer of promoter-*lacZ* fusions harbouring plasmids in *P. aeruginosa* was carried out as described previously (Boes *et al.*, 2008) modified by use of ST18 (Thoma and Schobert, 2009). Constructed plasmids were transferred into *P. aeruginosa* PAO1 wt and PAO1 Δ*sahH* strain. In these strains parts of the mini-CTX-*lacZ* vector containing the tetracycline resistance cassette (Tc^r) were not deleted. For the transfer of pME6013 constructs electroporation (settings: 25 µF, 200 Ω, 2.5 kV on a electroporator 2510 (Eppendorf)) was used as described previously (Choi *et al.*, 2006).

4.4.4 β-Galactosidase assay of transcriptional and translational promoter-*lacZ* reporter gene fusions

β-Galactosidase assays were performed as outlined before in detail (Boes *et al.*, 2008; Eschbach *et al.*, 2004), and activity was reported in Miller units (Miller, 1992).

To analyse the activation and presence of riboswitches and the impact of ribavirin the AMC promoter constructs were incubated under aerobic conditions. Bacteria were grown in M9 minimal medium supplemented with 50 µM methionine and 50 µg/ml ribavirin (204 µM), respectively. Cultures were grown in 1.5 ml medium in wells of a 24 well plate (Thermo Fisher Scientific) and sealed with sealing film Breath-easy (Roth) for gas exchange and shaken at 300 rpm (Thermo Shaker PST-60 HL-4, Lab4You) at 37°C. OD_{578} was measured using Varioskan Flash (Thermo Scientific) and a conventional photometer Ultrospec (Amersham Biosciences). Bacteria were harvested when reaching a similar OD_{578} of 0.300 ± 0.05.

4.4.5 Construction of expression vector pET14b*::sahH*

The *sahH* expression vector was constructed using the *sahH* gene from *P. aeruginosa* PAO1. For PCR amplification primers oAW192 and oAW193 were used (for details see Supplemental Table 28) and cloned in the multiple cloning site (MCS) of pET14b (Novagen) between *Nde*I and *Bam*HI restriction sites.

4.4.6 Recombinant SahH production and purification

For protein production BL21 (DE3) containing the pET14b*::sahH* plasmid was cultivated at 37°C in 500 ml LB medium supplemented with 100 µg/ml carbenicillin until an OD_{578} of 0.6. Then protein production was induced by addition of 50 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were further cultivated for 18 h at 17°C. The cells were harvested by centrifugation and stored at -20°C.

All protein purification steps were performed at 4°C. Harvested *E. coli* cells were resuspended in buffer A [50 mM Tris-HCl (pH 7.5), 300 mM NaCl] containing 15 mM imidazole and 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by two French press passages at 1000 p.s.i. (68947.57 hPa) and afterwards centrifuged for 60 min at 175 000 g. The supernatant was applied to 1.5 ml of Ni Sepharose™ 6 Fast Flow (GE Healthcare) equilibrated with buffer A.

After extensive washing with buffer A, His-SahH was eluted with buffer A containing 300 mM imidazole. NAP-25 column (GE Healthcare) was used for buffer exchange.

N-terminal His tag was removed with thrombin using the Thrombin Cleavage Capture Kit (Novagen) according to the manufacturer's instructions. Cleaved SahH was separated from His-SahH by 1 ml of equilibrated Ni Sepharose^{TM} 6 Fast Flow. The protein concentration of the flow through was routinely determined with Bradford reagent (Sigma-Aldrich), using BSA as standard. The purified SahH protein was stored at -20°C in buffer A with 10% glycerol.

4.4.7 SahH detection via Western blot analysis

Protein samples were separated in 12 % SDS-PAGE gel and stained with Coomassie blue. Western blot analyses were performed using standard protocols (Sambrook and Russell, 2001). Briefly, protein samples of purified SahH, *P. aeruginosa* culture supernatant, CF- Sputum samples (Supplemental Table 29) and blood samples were separated by SDS-PAGE and electro-blotted onto a polyvinylidene fluoride (PVDF) membrane (Schleicher & Schuell) using Towbin buffer (Towbin *et al.*, 1979). The membranes were then incubated for 60 min at room temperature in blocking solution (TBS [50 mM Tris-HCl, 150 mM NaCl] containing 3% BSA), after intensive washing with TBS-T containing 0.1% Tween 20, followed by an 1 h incubation with polyclonal rabbit antibody against *P. aeruginosa* SahH (Davids biotechnology). The membrane was incubated for 1 h with 1:5000 alkaline phosphatase conjugated goat anti-rabbit IgG polyclonal secondary antibody (Thermo Scientific). Immune complexes were visualised by the conversion of 5-brom-4-chlor-3-indoxyl phosphate (BCIP) (Merck) in the presence of nitro blue tetrazolium (NBT) (Merck) and oxygen into 5,5'-dibromo-4,4'-dichloro indigo.

Anti-Pa SahH polyclonal rabbit antibodies were produced by Davids biotechnology. The antibodies were isolated from rabbit sera using 1.5 ml protein A & G resin (2:1 mixture) (GenScript). Briefly, rabbit serum was diluted in buffer B [150 mM NaCl, 20 mM Na_2HPO_4, pH 8.0] and bound to the resin. Antibodies were eluted and neutralized according to the manufacturer`s instructions. Protein concentration was determined as described above. Antibodies were stored at - 20°C until required and used at a concentration of 0.1 µg/ml.

4.4.8 SahH activity assay

In vitro SahH activity assay was performed as previously described (Cai *et al.*, 2007; Yuan *et al.*, 1996). The SahH reaction in the hydrolytic direction was investigated. SAH is converted into Ado and Hcy with the modification that experiments were conducted at 37°C with 0.11 µM His-SahH in a final volume of 1 ml. Furthermore no NAD^+ was added to the reaction. The generation of the yellow 5-thio-2-nitrobenzoate TNB^{2-} (extinction coefficient: 13 600 $M^{-1} \times cm^{-1}$) from 5,5'-dithiobis-(2-nitrobenzoic acid) DTNB (Fluka) during the reaction was monitored using a V-550 UV/Vis spectrophotometer (Jasco) at 412 nm and 37 °C. For the inhibition experiment SahH was incubated with 0 µM and 100 µM ribavirin (Sigma), and samples were taken after 0, 5, 10, 30, 75 and 120 min. 1 U of adenosine deaminase (Sigma-Aldrich) was added directly before the addition of the substrate solution containing S-adenosylhomocysteine (Sigma) and DTNB in 50 mM phosphate buffer pH 7.2.

4.5 Supplemental information

4.5.1 Blood purification

Briefly, blood sample was taken and mixed with heparin. Afterwards the blood sample was diluted 1:1 with 1 x PBS pH 7.5. A ficoll paque™ Plus (GE Healthcare) and histopaque 1119 (Sigma) gradient was used to separate erythrocytes, monocytes and serum.

4.5.2 Cystic fibrosis-sputum samples

CF-sputum samples were a generous gift of Michael Hogardt, Munich. Samples were stored at -80°C.

Table 26: Bacterial Strains

Bacterial strains	Genotype	Reference
P. aeruginosa:		
PAO1	Wild type	(Dunn and Holloway, 1971)
ST26	PAO1 Δ*sahH* (PA0432)	(Thoma, 2009)
AW07	PAO1 Δ*mtnP* (PA3004)	This study
AW12	PAO1 Δ*sahH* Δ*mtnP* (PA0432, PA3004)	This study
AW18	PAO1 Δ*metZ* (PA3107)	This study
AW28	PAO1 pME6013, Tc^r	This study
AW29	PAO1 pAW10, Tc^r	This study
AW31	PAO1 pAW12, Tc^r	This study
AW32	PAO1 pAW13, Tc^r	This study
AW36	PAO1 *attB*::(*lacZ*), Tc^r .PAO1 containing promoterless *lacZ* from mini-CTX-*lacZ*	This study
AW37	PAO1 *attB*::(P_{metK}-*lacZ*), Tc^r	This study
AW39	PAO1 *attB*::(P_{metH}-*lacZ*), Tc^r	This study
AW40	PAO1 *attB*::(P_{sahH}-*lacZ*), Tc^r	This study
AW78	PAO1 Δ*sahH* pME6013, Tc^r	This study
AW79	PAO1 Δ*sahH* pAW10, Tc^r	This study
AW81	PAO1 Δ*sahH* pAW12, Tc^r	This study
AW82	PAO1 Δ*sahH* pAW13, Tc^r	This study
AW85	PAO1 Δ*sahH attB*::(*lacZ*), Tc^r	This study
AW86	PAO1 Δ*sahH attB*::(P_{metK}-*lacZ*), Tc^r	This study
AW88	PAO1 Δ*sahH attB*::(P_{metH}-*lacZ*), Tc^r	This study
AW89	PAO1 Δ*sahH attB*::(P_{sahH}-*lacZ*), Tc^r	This study
E. coli:		
DH10B	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15* Δ*lacX74 deoR recA1 endA1 araD139* Δ*(ara, leu) 7697 galU galK λ⁻ rpsL nupG*	GibcoBRL (Invitrogen)
ST18	S17 λ*pir* Δ*hemA*	(Thoma and Schobert, 2009)
BL21 (DE3)	*F⁻ ompT hsdSB*(r_B^- m_B^-) *gal dcm* (DE3)	Novagen

Supplemental Material:

Table 27: Plasmids

Plasmid	Genotype	Reference
pEX18AP	Ap^r, *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS of pUC18	(Hoang *et al.*, 1998)
pPS858	Ap^r, Gm^r, source of gentamicin cassette	(Hoang *et al.*, 1998)
pFLP2	Ap^r, source of FLP recombinase	(Hoang *et al.*, 1998)
pST06	Ap^r Gm^r; pEX18Ap with 593 bp upstream *sahH*, Gm^r-gfp fragment from pPS858 *Sac*I digested, and 473 bp downstream of the coding region of *sahH* between *KpnI* and *XbaI*	(Thoma, 2009)
pMR01	Ap^r Gm^r; pEX18Ap with 751 bp upstream *metZ*, Gm^r-gfp fragment from pPS858 *Bam*HI digested, and 524 bp downstream of the coding region of *metZ* between *KpnI* and *XbaI*	This study
pAW03	Ap^r Gm^r; pEX18Ap with 752 bp upstream *mtnP*, Gm^r-gfp fragment from pPS858 *Bam*HI digested, and 522 bp downstream of the coding region of *mtnP* between *KpnI* and *XbaI*	This study
pET14b	Ap^r, N-terminal His Tag ®, thrombin site, T7 promoter, T7 terminator, pBR322 origin	Novagen
pAW04	Ap^r, pET14b with 1416 bp of the coding region of *sahH* between *Nde*I and *Bam*HI and N-terminal His Tag	This study
pME6013	Cloning vector for construction of translational *lacZ* fusions; Tc^r	(Schnider-Keel *et al.*, 2000)
mini-CTX- *lacZ*	self-proficient integration vector for construction of transcriptional *lacZ* fusions; Tc^r	(Becher and Schweizer, 2000)
pAW10	pME6013 containing a 659 bp *EcoRI-PstI* fragment, encoding 500 bp upstream of the *metK* translational start and 159 bp of the coding region, Tc^r; pME6013::*metK*	This study
pAW12	pME6013 containing a 650 bp *EcoRI-PstI* fragment, encoding 500 bp upstream of the *metH* translational start and 150 bp of coding region, Tc^r; pME6013::*metH*	This study
pAW13	pME6013 containing a 659 bp *EcoRI- EcoRI* fragment, encoding 500 bp upstream of the *sahH* translational start and 159 bp of and coding region, Tc^r, orientation was confirmed via PCR; pME6013::*sahH*	This study
pAW17	mini-CTX-lacZ containing a 659 bp *EcoRI-PstI* fragment, encoding 500 bp upstream of the *metK* translational start and 159 bp of the coding region, Tc^r ;mini-CTX- *lacZ*::*metK*	This study
pAW19	mini-CTX-lacZ containing a 650 bp *EcoRI-PstI* fragment , encoding 500 bp upstream of the *metH* translational start and 150 bp of coding region, Tc^r; mini-CTX- *lacZ*::*metH*	This study
pAW20	mini-CTX-lacZ containing a 659 bp *EcoRI- EcoRI* fragment, encoding 500 bp upstream of the *sahH* translational start and 159 bp of and coding region, Tc^r, orientation was confirmed via PCR; mini-CTX- *lacZ*::*sahH*	This study

Table 28: Primer (restriction sites are underlined)

Primer	Primer sequence (5' - 3')	Restriction site	Construct
pST06 *sahH*:			
sahHP1	GGAGTACCACCAGGGCGCTGCACATC	*Kpn*I	upstream fragment
sahHP2	CGAGCTCTGACAGCGCTCATGCGTAA	*Sac*I	upstream fragment
sahHP3	CGAGCTCCGGACACCTATCGCTACTA	*Sac*I	downstream fragment
sahHP4	GCTCTAGATGGCTGCAGGTTGGTGAT	*Xba*I	downstream fragment
pMR01 *metZ*:			
oAW208	GGGGTACCTCGTTGTCGGCAAGCACTA	*Kpn*I	upstream fragment
oAW207	CGGGATCCGCCATCCTCAGCATGAGTT	*Bam*HI	upstream fragment
oAW152	CGGGATCCGCCGCTCTCCCCTTTGTCA	*Bam*HI	downstream fragment
oAW153	GCTCTAGACCAGGCGGCGCGGAAGAAA	*Xba*I	downstream fragment
pAW03 *mtnP*:			
oAW154	GGGGTACCGCCTATGCGCTTGTTCCGT	*Kpn*I	upstream fragment
oAW155	CGGGATCCGGCGTTGCACGATGGCATT	*Bam*HI	upstream fragment
oAW162	CGGGATCCCTCCCTCAATCAATCAG	*Bam*HI	downstream fragment
oAW163	GCTCTAGAAGCATTTTCCCGGTCAC	*Xba*I	downstream fragment
pAW04:			
oAW192	GGAATTCCATATGATGAGCGCTGTCAT	*Nde*I	pET-14b::*sahH*
oAW193	CGCGGATCCTTAGTAGCGATAGGTGTC	*Bam*HI	pET-14b::*sahH*
lacZ constructs:			
AW233	CGGAATTCCGATGCCGAGGTCGTACC	*EcoRI*	P_{metK}
AW234	TGCACTGCAGTGATTCAGTCGCAGCGAGTC	*PstI*	P_{metK}
AW237	CGGAATTCTCGCCGGCCTGAAGGGCC	*EcoRI*	P_{metH}
AW238	TGCACTGCAGTGCATGCCGCCATCGAGGAT	*PstI*	P_{metH}
oAW243	CGGAATTCCAGGGATAGCCATTGGCTGGC	*EcoRI*	P_{sahH}
oAW244	CGGAATTCGCCTTTCAGCGGCTGCTG	*EcoRI*	P_{sahH}

Table 29: CF-sputum samples (from Michael Hogardt, Munich)

Lane SDS-PAGE	Number	Patient code	Date (Day/month/year)
1	132	BM_000 132	15/04/11
2	177	CM_000 177	25/05/11
3	025	CM_000 025	21/01/11
4	154	CM_000 154	11/05/11
5	090	BM_000 090	17/03/11
6	153	CM_000 153	11/05/11
7	369	C10_000 369	09/12/10
8	373	B10_000 373	15/11/10
9	091	BM_000 091	17/03/11
10	089	BM_000 089	17/03/11
11	372	C10_000 372	15/12/10
12	368	C10_000 368	09/12/10
13	148	CM_000 148	05/09/11

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5 Outlook

The development of ASM-Chelex allows further interesting investigations. Little modifications have to be implemented such as increased phosphate addition to overcome the phosphate limitation, induced by Chelex to get closer to *ex vivo* conditions. Moreover, the incubation times of the *P. aeruginosa* strains have to be optimized to reach an state of metabolic inactivity close to the *ex vivo* conditions. It would be also important to search for further iron chelators, with a high affinity for iron and reduced ability to chelate other substances, which is not metabolized by *P. aeruginosa*.

Furthermore, different characteristic *P. aeruginosa* isolates from different CF patients should be analysed in ASM-Chelex. This would allow the determination of the more general answers of *P. aeruginosa* towards the CF environment. Moreover, strain specific answers could also be elucidated and the underlying adaptation strategies of the respective strains could be identified. In general, it is of great importance to obtain more *ex vivo* transcriptome data and compare them to *in vitro* ASM-Chelex data. Furthermore, ASM-Chelex is suitable for the analysis of new antibiotic targets and new antibiotic substances. Evolutionary adaptations could be analysed in this model system. Interactions between different *P. aeruginosa* strains and with other colonizing pathogens could be further investigated and new insights into the pathogenesis could be derived.

In order to prove the increased iron scavenger ability and the resulting increased intracellular iron content of *P. aeruginosa* mutant strain PAO1 Δ*metF* certain experiments need to be conducted. The intracellular iron concentration of PAO1 Δ*metF* should be determined. The rate of iron-sulphur cluster proteins should be investigated. It would be interesting to analyse the growth of PAO1 Δ*metF* in the presence of high iron concentrations with and without sufficient methionine supplementation.

For further investigations of *P. aeruginosa* SahH as antibiotic target crystallization would be the next step to determine structural differences compared to the human SAHH. This would allow structure dependent inhibitor design and new functional inhibitors could be determined.

The mode of action of ribavirin against *P. aeruginosa* should be further investigated.

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7 Appendix

7.1 List of figures

7.2 List of tables

7.3 Appendix A

Table 30: Microarray analysis of iron regulated genes in ASM and ASM-Chelex compared to *ex vivo* patients' samples and comparison of both media

Differentially regulated genes (pfp < 0.05) of the clinical *Pseudomonas aeruginosa* strains CFCZ and CF1 - 4 grown in ASM (A) and ASM-Chelex (ACh) in comparison with *ex vivo* (EV) sputum samples. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated genes in artificial sputum medium ASM (A) compared to *ex vivo* (A / EV), ASM-Chelex compared to *ex vivo* (ACh / EV) and ASM-Chelex compared to ASM (ACh / A).

Gene ID[a]	Gene name[a]	Function[a]	PCFC[a,b]	FC A / EV	FC ACh / EV	FC ACh / A
1996806-1997509		unknown function	UGS	6,5	8,6	
1997509-1996806		unknown function	UGS	9,4	8,7	
3112877-3112150		unknown function	UGS		5,3	5,6
517462-518083		unknown function	UGS	3,9		
630527-629884		unknown function	UGS	5,0	7,8	
PA0044	*exoT*	exoenzyme T	SF	-13,5	-13,2	
PA0062		hypothetical protein	HUU			4,0
PA0070		hypothetical protein	MP			-5,1
PA0075	*pppA*	PppA	PE ; PSE			-3,4
PA0083		conserved hypothetical protein	HUU			-6,6
PA0084		conserved hypothetical protein	HUU			-4,1
PA0085	*hcp1*	Hcp1	SF			-7,0
PA0105	*coxB*	cytochrome c oxidase, subunit II	EM	23,3	26,2	
PA0106	*coxA*	cytochrome c oxidase, subunit I	EM	35,7	42,8	
PA0107		conserved hypothetical protein	EM	25,0	33,2	
PA0108	*coIII*	cytochrome c oxidase, subunit III	EM	43,7	48,5	
PA0110		hypothetical protein	HUU	7,3	8,6	
PA0111		hypothetical protein	HUU	17,6	19,5	
PA0113		probable cytochrome c oxidase assembly factor	EM	9,9	9,5	
PA0131		hypothetical protein	HUU	4,6		
PA0132		beta-alanine--pyruvate transaminase	ABM	5,4	5,0	
PA0140	*ahpF*	alkyl hydroperoxide reductase subunit F	AP	-14,6	-13,9	
PA0150		probable transmembrane sensor	MP ; TR	-7,9	-9,0	
PA0161		hypothetical protein	HUU	6,9		-5,6
PA0177		probable purine-binding C protein	AP ; C			-3,7
PA0179		probable two-component response regulator	C ; AP ; TCR	11,3		
PA0200		hypothetical protein	HUU	4,1		
PA0201		hypothetical protein	HUU	-4,9	-5,0	
PA0226		probable CoA transferase, subunit A	CC	95,1		-64,1
PA0227		probable CoA transferase, subunit B	CC	53,3		-42,6
PA0228	*pcaF*	beta-ketoadipyl CoA thiolase PcaF	FPM ; CC	55,6		-54,9
PA0229	*pcaT*	dicarboxylic acid transporter PcaT	MP ; TSM	5,6		-5,4
PA0230	*pcaB*	3-carboxy-cis,cis-muconate cycloisomerase	CC	6,6		-6,5
PA0231	*pcaD*	beta-ketoadipate enol-lactone hydrolase	CC	10,6		-10,2
PA0232	*pcaC*	gamma-carboxymuconolactone decarboxylase	CC	31,1		-19,6
PA0269		conserved hypothetical protein	HUU		3,2	
PA0283	*sbp*	sulfate-binding protein precursor	TSM		-6,0	
PA0284		hypothetical protein	HUU		-5,8	
PA0316	*serA*	D-3-phosphoglycerate dehydrogenase	ABM	-6,3	-15,2	
PA0320		conserved hypothetical protein	HUU	-16,7		25,2
PA0327		hypothetical protein	HUU			6,0
PA0365		hypothetical protein	MP		4,8	
PA0382	*micA*	DNA mismatch repair protein MicA	DRR		-4,7	
PA0430	*metF*	5,10-methylenetetrahydrofolate reductase	ABM ; CIM		-5,5	
PA0439		probable oxidoreductase	PE	27,3	5,5	-5,0
PA0440		probable oxidoreductase	PE	5,2		
PA0441	*dht*	dihydropyrimidinase	NM	8,6		
PA0444		N-carbamoyl-beta-alanine amidohydrolase	NM	22,3	6,5	-3,4
PA0447	*gcdH*	glutaryl-CoA dehydrogenase	FPM ; ABM ;		7,1	3,6
PA0459		probable ClpA/B protease ATP binding subunit	CC	4,3	6,7	
PA0461		conserved hypothetical protein	TPD	5,8	5,2	
PA0465	*creD*	inner membrane protein CreD	HUU	-76,3	-76,3	
PA0466		hypothetical protein	MP	-29,5	-29,6	
PA0472	*fiuR*	probable sigma-70 factor, ECF subfamily	HUU	-9,0		

PA0484		conserved hypothetical protein	TR	5,3	5,8	
PA0505		hypothetical protein	HUU	7,9		
PA0524	*norB*	nitric-oxide reductase subunit B	HUU	-6,9	-6,9	
PA0546	*metK*	methionine adenosyltransferase	EM	-8,4	-8,8	
PA0547		probable transcriptional regulator	ABM ; CIM		-4,9	
PA0576	*rpoD*	sigma factor RpoD	TR		-4,9	
PA0577	*dnaG*	DNA primase	TR		-4,5	
PA0578		conserved hypothetical protein	DRR		-7,7	
PA0588		conserved hypothetical protein	HUU		5,2	
PA0612	*ptrB*	repressor, PtrB	HUU			8,7
PA0613		hypothetical protein	TR			6,6
PA0614		hypothetical protein	HUU		14,4	6,8
PA0622		probable bacteriophage protein	HUU		6,2	
PA0623		probable bacteriophage protein	RPT		7,6	8,2
PA0624		hypothetical protein	RPT		10,6	7,3
PA0627		conserved hypothetical protein	RPT		7,1	7,6
PA0631		hypothetical protein	RPT		5,7	5,7
PA0633		hypothetical protein	RPT		5,5	5,2
PA0634		hypothetical protein	RPT		11,4	8,7
PA0635		hypothetical protein	RPT		29,2	22,6
PA0636		hypothetical protein	RPT		6,4	5,3
PA0637		conserved hypothetical protein	RPT		6,2	5,4
PA0638		probable bacteriophage protein	RPT		6,2	
PA0639		conserved hypothetical protein	RPT		6,0	5,6
PA0647		hypothetical protein	RPT		7,2	4,0
PA0648		hypothetical protein	RPT		18,2	19,3
PA0656		probable HIT family protein	PE	8,9	11,1	
PA0672	*hemO*	heme oxygenase	BC	-133,3	-48,5	
PA0674	*vreA*	VreA	TR	-21,4		8,7
PA0730		probable transferase	PE			4,8
PA0737		hypothetical protein	HUU	-8,3		5,7
PA0738		conserved hypothetical protein	MP	-8,4		4,3
PA0745		probable enoyl-CoA hydratase/isomerase	PE	4,8	7,0	
PA0746		probable acyl-CoA dehydrogenase	PE		6,0	
PA0767	*lepA*	GTP-binding protein LepA	TPD ; PSE		-4,9	
PA0781		hypothetical protein	HUU	-14,9		5,0
PA0782	*putA*	proline dehydrogenase PutA	ABM	-6,1		
PA0795	*prpC*	citrate synthase 2	CIM ; CC	6,4	6,6	
PA0802		hypothetical protein	MP	-6,1		
PA0805		hypothetical protein	HUU	-7,5		
PA0807		AmpDh3	ARS		13,2	17,7
PA0808		hypothetical protein	HUU		7,4	7,0
PA0848		probable alkyl hydroperoxide reductase	AP ; PE	-11,7	-5,7	
PA0849	*trxB2*	thioredoxin reductase 2	NM	-8,4	-6,9	
PA0850		hypothetical protein	HUU	4,8	7,7	
PA0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase	ABM	13,7	29,3	
PA0866	*aroP2*	aromatic amino acid transport protein AroP2	TSM	6,4	21,6	
PA0867	*mliC*	membrane-bound lysozyme inhibitor of c-type l	HUU ; AP		5,0	
PA0870	*phhC*	aromatic amino acid aminotransferase	ABM		5,8	
PA0871	*phhB*	pterin-4-alpha-carbinolamine dehydratase	ABM	4,5	12,8	
PA0872	*phhA*	phenylalanine-4-hydroxylase	ABM		9,8	
PA0876		probable transcriptional regulator	TR	6,2	7,8	
PA0887	*acsA*	acetyl-coenzyme A synthetase	CC ; CIM	15,0	7,3	
PA0908		hypothetical protein	HUU		13,5	6,9
PA0910		hypothetical protein	HUU		21,4	11,9
PA0911		hypothetical protein	HUU		13,7	7,5
PA0921		hypothetical protein	MP	22,3	30,8	
PA0929		two-component response regulator	TSM ; TCR	-22,6		10,0
PA0930		two-component sensor	TSM ; TCR	-8,1		
PA0959		hypothetical protein	HUU		4,9	
PA0996	*pqsA*	probable coenzyme A ligase	BC	5,3	34,3	6,4
PA0997	*pqsB*	Homologous to beta-keto-acyl-acyl-carrier p.s.$^{+}$	BC		30,6	14,7
PA0998	*pqsC*	Homologous to beta-keto-acyl-acyl-carrier p.s.$^{+}$	BC		39,0	13,1
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	BC	4,1	34,7	8,4
PA1000	*pqsE*	Quinolone signal response protein	BC		23,5	12,9
PA1001	*phnA*	anthranilate synthase component I	AP		34,1	19,5
PA1002	*phnB*	anthranilate synthase component II	ABM ; AP		26,7	10,9
PA1041		probable o.m. protein precursor	MP ; TSM	9,6	11,3	
PA1077	*flgB*	flagellar basal-body rod protein FlgB	CLC ; MA		-6,8	
PA1078	*flgC*	flagellar basal-body rod protein FlgC	CLC ; MA	-5,9	-20,2	-3,4
PA1079	*flgD*	flagellar basal-body rod protein FlgD	CLC ; MA	-4,8	-11,7	
PA1080	*flgE*	flagellar hook protein FlgE	CLC ; MA	-7,4	-35,6	-4,8
PA1081	*flgF*	flagellar basal-body rod protein FlgF	CLC ; MA	-6,9	-8,2	
PA1082	*flgG*	flagellar basal-body rod protein FlgG	CLC ; MA		-7,5	
PA1083	*flgH*	flagellar L-ring protein precursor FlgH	CLC ; MA		-5,2	
PA1084	*flgI*	flagellar P-ring protein precursor FlgI	CLC ; MA		-6,7	
PA1134		Predicted thiol-disulphide oxidoreductase	HUU		5,1	6,2
PA1135		conserved hypothetical protein	HUU	24,9	73,2	

PA1137		probable oxidoreductase	PE	5,1		-3,6
PA1159		probable cold-shock protein	TR ; AP		5,6	
PA1172	*napC*	cytochrome c-type protein NapC	EM	5,6		
PA1173	*napB*	cytochrome c-type protein NapB precursor	EM	6,9	5,0	
PA1174	*napA*	periplasmic nitrate reductase protein NapA	EM	28,3	16,0	
PA1175	*napD*	NapD protein of periplasmic nitrate reductase	EM	8,0	4,9	
PA1176	*napF*	ferredoxin protein NapF	EM	15,0	12,3	
PA1177	*napE*	periplasmic nitrate reductase protein NapE	EM	26,8	35,0	
PA1178	*oprH*	PhoP/Q, low Mg2+ inducible o.m. prot. H1 prc.	MP ; AP		5,6	
PA1179	*phoP*	two-component response regulator PhoP	TR ; TCR		7,6	
PA1180	*phoQ*	two-component sensor PhoQ	TCR		9,4	
PA1183	*dctA*	C4-dicarboxylate transport protein	TSM	-6,8		5,9
PA1190		conserved hypothetical protein	MP		6,1	
PA1193		hypothetical protein	HUU		-6,2	
PA1198		conserved hypothetical protein	HUU	5,9	7,2	
PA1283		probable transcriptional regulator	TR	4,2	9,6	
PA1300		probable sigma-70 factor, ECF subfamily	TR	-42,2	-37,5	
PA1301		probable transmembrane sensor	MP ; TR	-14,5	-12,0	
PA1302		probable heme utilization protein precursor	MP ; TSM	-5,6	-5,6	
PA1327		probable protease	PE		6,4	
PA1337	*ansB*	glutaminase-asparaginase	ABM	8,9	7,0	
PA1338	*ggt*	gamma-glutamyltranspeptidase precursor	ABM ; AP	5,5		
PA1339		amino acid ABC transporter ATP binding protein	TSM	6,7	4,7	
PA1340		amino acid ABC transporter membrane protein	TSM	10,6	9,4	
PA1341		amino acid ABC transporter membrane protein	MP ; TSM	9,5	7,6	
PA1342		probable binding protein component of ABC	TSM	12,1	9,5	
PA1348		hypothetical protein	HUU	4,7	6,6	
PA1404		hypothetical protein	HUU		9,1	
PA1441		hypothetical protein	HUU	-7,6	-14,6	
PA1471		hypothetical protein	HUU			6,7
PA1540		conserved hypothetical protein	MP	-16,5	-8,7	
PA1542		hypothetical protein	HUU	3,9	12,8	
PA1555		probable cytochrome c	EM		12,4	5,9
PA1556		probable cytochrome c oxidase subunit	EM		8,5	5,9
PA1557		probable cytochrome oxidase subunit (cbb3-typ.)	EM	4,2	10,6	
PA1571		hypothetical protein	HUU		5,3	
PA1591		hypothetical protein	MP		-6,9	
PA1606		hypothetical protein	HUU		36,7	37,2
PA1608		probable Chemotaxis transducer	AP ; C	-5,1	-5,1	
PA1617		probable AMP-binding enzyme	PE	4,2	5,6	
PA1688		hypothetical protein	HUU		-8,4	
PA1698	*popN*	Type III secretion o.m. protein PopN precursor	MP ; PSE		-4,5	
PA1699		conserved hypothetical protein in type III secr.	PSE	-7,5	-7,5	
PA1700		conserved hypothetical protein in type III secr.	PSE	-9,0	-10,3	
PA1701		conserved hypothetical protein in type III secr.	PSE	-9,5	-9,5	
PA1702		conserved hypothetical protein in type III secr.	PSE	-18,9	-18,5	
PA1706	*pcrV*	type III secr. protein PcrV	PSE	-9,3	-9,4	
PA1707	*pcrH*	regulatory protein PcrH	SF ; PSE	-9,0	-10,8	
PA1708	*popB*	translocator protein PopB	PSE	-15,7	-16,1	
PA1710	*exsC*	exoenzyme S synthesis protein C precursor.	TPD ; PSE	-4,6	-6,2	
PA1711	*exsE*	ExsE	HUU ; TR	-8,5	-19,2	
PA1712	*exsB*	exoenzyme S synthesis protein B	TPD ; PSE	-16,3	-20,3	
PA1713	*exsA*	transcriptional regulator ExsA	PSE ; TR	-5,6	-5,3	
PA1714	*exsD*	ExsD	HUU	-9,4	-12,7	
PA1715	*pscB*	type III export apparatus protein	PSE	-16,2	-16,2	
PA1716	*pscC*	Type III secr. o.m. protein PscC precursor	PSE	-12,7	-14,7	
PA1717	*pscD*	type III export protein PscD	PSE	-7,6	-8,1	
PA1718	*pscE*	type III export protein PscE	PSE	-12,6	-12,6	
PA1722	*pscI*	type III export protein PscI	PSE	-7,2	-7,2	
PA1728		hypothetical protein	HUU		6,6	
PA1789		hypothetical protein	HUU	4,8	5,2	
PA1842		hypothetical protein	HUU		-4,9	
PA1843	*metH*	methionine synthase	ABM		-6,0	
PA1911	*femR*	sigma factor regulator, FemR	MP ; TR	-7,4	-5,1	
PA1912	*femI*	ECF sigma factor, FemI	TR	-11,1	-7,7	
PA1921		hypothetical protein	HUU			11,6
PA1922		probable TonB-dependent receptor	TSM	-8,8		15,6
PA1924		hypothetical protein	HUU	-7,0		7,3
PA1925		hypothetical protein	HUU	-10,5		15,7
PA1927	*metE*	cobalamin-independent methionine synthase	ABM	-13,4	-13,7	
PA1985	*pqqA*	pyrroloquinoline quinone biosynthesis protein	BC		26,8	17,8
PA1999	*dhcA*	dehydrocarnitine CoA transferase, subunit A	ABM ; CC		8,8	6,4
PA2000	*dhcB*	dehydrocarnitine CoA transferase, subunit B	ABM ; CC		7,3	4,8
PA2004		conserved hypothetical protein	MP			4,3
PA2008	*fahA*	fumarylacetoacetase	CC		5,0	
PA2024		probable ring-cleaving dioxygenase	PE	8,2	13,6	
PA2026		conserved hypothetical protein	MP			-4,0
PA2027		hypothetical protein	HUU	203,5		-200,0

PA2033		hypothetical protein	HUU	-16,4	-12,5	
PA2034		hypothetical protein	HUU	-26,8	-24,3	
PA2110		hypothetical protein	HUU		7,7	8,6
PA2111		hypothetical protein	HUU		14,0	14,6
PA2112		conserved hypothetical protein	HUU		13,2	13,3
PA2114		probable major facilitator superfamily transporter	MP ; TSM		12,7	14,7
PA2116		conserved hypothetical protein	HUU		16,1	46,2
PA2134		hypothetical protein	HUU		16,3	
PA2143		hypothetical protein	HUU	6,3	11,0	
PA2146		conserved hypothetical protein	HUU	19,5	37,3	
PA2159		conserved hypothetical protein	HUU		5,2	
PA2166		hypothetical protein	HUU	21,9	28,9	
PA2170		hypothetical protein	HUU	5,9	8,8	
PA2171		hypothetical protein	HUU		10,4	
PA2172		hypothetical protein	HUU		7,0	
PA2173		hypothetical protein	HUU	6,9	23,9	
PA2174		hypothetical protein	HUU		7,9	
PA2182		hypothetical protein	HUU	5,9	9,0	
PA2190		conserved hypothetical protein	HUU		13,3	
PA2231	*pslA*	PslA	CLC	4,1		
PA2232	*pslB*	PslB	CLC	3,8		
PA2247	*bkdA1*	2-oxoisovalerate dehydrogenase (alpha subunit)	ABM		12,7	
PA2248	*bkdA2*	2-oxoisovalerate dehydrogenase (beta subunit)	ABM		8,2	
PA2249	*bkdB*	branched-chain alpha-keto acid dehydrogenase	ABM		10,0	
PA2317		probable oxidoreductase	PE	9,6		-8,2
PA2318		hypothetical protein	HUU	15,1		-4,2
PA2321		gluconokinase	EM ; CC	-6,2	-6,6	
PA2331		hypothetical protein	MP		-5,7	
PA2364		hypothetical protein	HUU	5,7		
PA2365		conserved hypothetical protein	HUU	7,7		-4,1
PA2375		hypothetical protein	MP		8,0	
PA2378		probable aldehyde dehydrogenase	PE	4,9	4,7	
PA2379		probable oxidoreductase	PE		5,4	
PA2381		hypothetical protein	HUU	6,7	34,6	5,2
PA2382	*lldA*	L-lactate dehydrogenase	EM		-5,0	
PA2384		hypothetical protein	HUU	-17,8	-15,4	
PA2385	*pvdQ*	3-oxo-C12-homoserine lactone acylase PvdQ	AP	-11,4	-11,4	
PA2386	*pvdA*	L-ornithine N5-oxygenase	AP	-62,5	-62,5	
PA2393		probable dipeptidase precursor	CIM	-9,2	-9,2	
PA2394	*pvdN*	PvdN	AP	-20,4	-20,7	
PA2395	*pvdO*	PvdO	AP	-6,6	-6,6	
PA2396	*pvdF*	pyoverdine synthetase F	SF ; AP	-18,8	-24,9	
PA2397	*pvdE*	pyoverdine biosynthesis protein PvdE	MP ; AP	-17,8	-17,8	
PA2398	*fpvA*	ferripyoverdine receptor	TSM	-101,0	-80,0	
PA2399	*pvdD*	pyoverdine synthetase D	SF ; AP	-13,1	-14,0	
PA2400	*pvdJ*	PvdJ	AP	-8,6	-8,5	
PA2402		probable non-ribosomal peptide synthetase	PE	-13,3	-13,4	
PA2403		hypothetical protein	MP	-9,2	-5,8	
PA2404		hypothetical protein	MP	-12,1	-10,3	
PA2405		hypothetical protein	HUU	-22,1	-13,1	
PA2406		hypothetical protein	HUU	-13,5	-13,4	
PA2407		probable adhesion protein	MA	-28,2	-28,5	
PA2408		probable ATP-binding component of ABC transp.	TSM	-6,3	-6,8	
PA2409		probable permease of ABC transporter	MP ; TSM	-10,5	-7,3	
PA2411		probable thioesterase	AP ; PE	-20,0	-20,0	
PA2412		conserved hypothetical protein	HUU	-29,0	-25,9	
PA2413	*pvdH*	PvdH	AP	-6,2	-6,0	
PA2424	*pvdL*	PvdL	AP	-7,9	-7,9	
PA2426	*pvdS*	sigma factor PvdS	TR	-71,9	-37,6	
PA2427		hypothetical protein	HUU			5,2
PA2428		hypothetical protein	HUU		45,3	44,9
PA2433		hypothetical protein	HUU	18,7	39,8	
PA2442	*gcvT2*	glycine cleavage system protein T2	CIM ; ABM			4,9
PA2444	*glyA2*	serine hydroxymethyltransferase	ABM		5,8	
PA2444	*glyA2*	serine hydroxymethyltransferase	ABM			27,8
PA2445	*gcvP2*	glycine cleavage system protein P2	CIM ; ABM			23,1
PA2446	*gcvH2*	glycine cleavage system protein H2	ABM			16,2
PA2467	*foxR*	Anti-sigma factor FoxR	MP ; TR	-15,5	-8,6	
PA2468	*foxI*	ECF sigma factor FoxI	TR	-7,9	-4,9	
PA2476	*dsbG*	thiol:disulphide interchange protein DsbG	TPD ; CHP			4,3
PA2478		probable thiol:disulphide interchange protein	MP ; PE			4,8
PA2501		hypothetical protein	MP	13,1	7,6	
PA2504		hypothetical protein	HUU		6,5	
PA2507	*catA*	catechol 1,2-dioxygenase	CC	182,0		-103,1
PA2508	*catC*	muconolactone delta-isomerase	CC	159,8		-56,8
PA2509	*catB*	muconate cycloisomerase I	CC	55,1		-51,5
PA2511		probable transcriptional regulator	TR	21,8		-14,1
PA2512	*antA*	anthranilate dioxygenase large subunit	CC	102,5		-80,0

PA2513	*antB*	anthranilate dioxygenase small subunit	CC	241,7		-65,8
PA2514	*antC*	anthranilate dioxygenase reductase	CC	83,5		-80,0
PA2519	*xylS*	transcriptional regulator XylS	TR	5,3		-4,2
PA2552		probable acyl-CoA dehydrogenase	PE		4,9	
PA2555		probable AMP-binding enzyme	PE			5,7
PA2573		probable C transducer	AP ; C		4,9	
PA2618		hypothetical protein	TPD	5,2	5,9	
PA2622	*cspD*	cold-shock protein CspD	TR ; AP	10,0	11,2	
PA2624	*idh*	isocitrate dehydrogenase	EM		-5,1	
PA2635		hypothetical protein	HUU		6,6	5,4
PA2657		probable two-component response regulator	TR ; TCR			4,8
PA2658		hypothetical protein	HUU			9,0
PA2659		hypothetical protein	HUU		5,0	8,5
PA2662	*idh*	isocitrate dehydrogenase	EM	-5,1		
PA2679		hypothetical protein	HUU	6,2		
PA2682		conserved hypothetical protein	PE	22,2		-15,2
PA2686	*pfeR*	two-component response regulator PfeR	TR ; TCR	-5,1	-5,1	
PA2740	*pheS*	phenylalanyl-tRNA synthetase, alpha-subunit	TPD		-5,7	
PA2746		hypothetical protein	MP	8,3	24,0	
PA2747		hypothetical protein	HUU	12,6	8,4	
PA2762		hypothetical protein	HUU	11,7	7,7	
PA2779		hypothetical protein	HUU	4,6	8,5	
PA2803		hypothetical protein	HUU	12,7	12,1	
PA2804		hypothetical protein	HUU	10,5	10,5	
PA2840		probable ATP-dependent RNA helicase	TRD	-15,4	-25,4	
PA2850	*ohr*	organic hydroperoxide resistance protein	AP	-11,1	-11,1	
PA2881		probable two-component response regulator	TR ; TCR	5,4		4,1
PA2906		probable oxidoreductase	PE		-5,5	
PA2911		probable TonB-dependent receptor	MP ; TSM	-19,0	-19,6	
PA2912		probable ATP-binding component of ABC transp.	TSM	-13,8	-12,0	
PA2913		hypothetical protein	TSM	-10,6	-12,8	
PA2914		probable permease of ABC transporter	MP ; TSM	-10,4	-8,0	
PA2915		hypothetical protein	HUU	12,5	11,1	
PA2937		hypothetical protein	HUU	12,5	20,9	
PA3000	*aroP1*	aromatic amino acid transport protein AroP1	TSM		-5,8	
PA3038		probable porin	TSM	17,6	8,5	
PA3040		conserved hypothetical protein	HUU		5,2	
PA3049	*rmf*	ribosome modulation factor	TPD	10,0	12,6	
PA3080		hypothetical protein	HUU	5,8		
PA3118	*leuB*	3-isopropylmalate dehydrogenase	ABM	4,9		
PA3119		conserved hypothetical protein	HUU	5,6		-4,3
PA3119		conserved hypothetical protein	HUU			
PA3120	*leuD*	3-isopropylmalate dehydratase small subunit	ABM	13,3		-9,7
PA3120	*leuD*	3-isopropylmalate dehydratase small subunit	ABM			
PA3121	*leuC*	3-isopropylmalate dehydratase large subunit	ABM	12,1		-10,5
PA3123		conserved hypothetical protein	HUU	4,8	4,9	
PA3129		conserved hypothetical protein	HUU		-4,7	
PA3174		probable transcriptional regulator	TR			6,2
PA3183	*zwf*	glucose-6-phosphate 1-dehydrogenase	EM ; CC		-5,2	
PA3205		hypothetical protein	HUU		17,2	
PA3205		hypothetical protein	HUU			6,0
PA3216		probable aspartate-semialdehyde dehydrogenase	ABM	10,4	13,3	
PA3219		hypothetical protein	HUU		14,0	13,5
PA3231		probable aldolase	CIM ; CC	6,2	9,2	
PA3232		probable hydrolase	PE	4,9		
PA3234	*gltX*	glutamyl-tRNA synthetase	TPD	37,9	12,4	
PA3235		probable transcriptional regulator	TR	79,1	26,8	
PA3236		probable secretion protein	TSM	6,1		
PA3237		hypothetical protein	HUU	-13,9	-13,9	
PA3278		hypothetical protein	MP	6,1	5,7	
PA3280	*oprO*	Pyrophosphate-specific o.m. porin OprO prec.	TSM	-5,5	21,7	
PA3281		hypothetical protein	MP	-59,9	-18,3	
PA3282		hypothetical protein	HUU	-72,5	-14,6	
PA3283		conserved hypothetical protein	HUU	-66,7	-13,2	5,0
PA3284		hypothetical protein	HUU	-29,8	-7,1	
PA3308	*hepA*	RNA helicase HepA	TRD		-4,6	
PA3347		hypothetical protein	HUU		5,3	
PA3362		hypothetical protein	MP	6,1		
PA3363	*amiR*	aliphatic amidase regulator	CC ; TR	5,4		
PA3365		probable chaperone	CHP	7,3	-7,3	
PA3366	*amiE*	aliphatic amidase	CC	8,9		
PA3368		probable acetyltransferase	PE		6,2	5,5
PA3369		hypothetical protein	MP	11,6	9,8	
PA3371		hypothetical protein	HUU	5,6	4,8	
PA3382	*phnE*	phosphonate transport protein PhnE	MP ; TSM		9,3	9,6
PA3383		binding protein component of ABC phosphonate t.	TSM		51,3	51,3
PA3384	*phnC*	ATP-binding component of ABC phosphonate t.	TSM		9,7	9,7
PA3397	*fpr*	ferredoxin--NADP+ reductase	BC ; EM	-6,9	-10,0	

PA3399		hypothetical protein	HUU	4,5		
PA3407	*hasAp*	heme acquisition protein HasAp	TSM	-250,0	-227,3	
PA3408	*hasR*	Haem uptake o.m. receptor HasR precursor	TSM	-7,0	-7,0	
PA3415		probable dihydrolipoamide acetyltransferase	EM	12,0	16,7	
PA3416		probable pyruvate dehydrogenase E1 component, beta chain	EM	8,2	9,3	
PA3417		probable pyruvate dehydrogenase E1 component, alpha subunit	EM	5,8	9,4	
PA3418	*ldh*	leucine dehydrogenase	ABM	10,4	16,4	
PA3431		conserved hypothetical protein	MP	-95,2	-105,3	
PA3432		hypothetical protein	MP	-65,8	-61,7	
PA3441		probable molybdopterin-binding protein	TSM	-5,1		
PA3445		conserved hypothetical protein	HUU	-6,0	-6,0	
PA3446		conserved hypothetical protein	HUU	-7,9	-8,9	
PA3450		probable antioxidant protein	AP	-8,8	-11,5	
PA3451		hypothetical protein	HUU		8,8	
PA3465		conserved hypothetical protein	MP	5,9	5,1	
PA3496		hypothetical protein	HUU		14,0	
PA3530	*bfd*	bacterioferritin-associated ferredoxin	HUU			10,2
PA3531	*bfrB*	bacterioferritin	TSM ; AP	8,4		-4,3
PA3568		probable acetyl-coa synthetase	PE	8,8	8,3	
PA3569	*mmsB*	3-hydroxyisobutyrate dehydrogenase	CC	8,9	15,6	
PA3570	*mmsA*	methylmalonate-semialdehyde dehydrogenase	ABM ; CC	3,9	4,7	
PA3572		hypothetical protein	HUU	3,8	6,8	
PA3575		hypothetical protein	MP	7,8	13,0	
PA3578		conserved hypothetical protein	HUU			5,1
PA3586		probable hydrolase	PE	-5,6		4,9
PA3598		conserved hypothetical protein	HUU	-17,2	-10,8	
PA3600		conserved hypothetical protein	TPD	-161,3		57,3
PA3601		conserved hypothetical protein	TPD	-54,9		29,0
PA3615		hypothetical protein	HUU	3,8		
PA3622	*rpoS*	sigma factor RpoS	TR	4,6	9,8	
PA3628		probable esterase	PE	4,7		
PA3629	*adhC*	alcohol dehydrogenase class III	CIM	5,8		
PA3638		conserved hypothetical protein	HUU		-5,0	
PA3656	*rpsB*	30S ribosomal protein S2	TPD		-6,7	
PA3662		hypothetical protein	HUU	16,7	12,4	
PA3684		hypothetical protein	HUU	4,5	4,9	
PA3688		hypothetical protein	HUU	8,0	9,3	
PA3700	*prfB*	peptide chain release factor 2	TPD		-6,1	
PA3723		probable FMN oxidoreductase	PE	15,4	25,1	
PA3731		conserved hypothetical protein	HUU			-3,7
PA3741		hypothetical protein	HUU	-23,6	-36,2	
PA3742	*rplS*	50S ribosomal protein L19	TPD		-6,7	
PA3743	*trmD*	tRNA (guanine-N1)-methyltransferase	TRD		-5,2	
PA3768		probable metallo-oxidoreductase	PE	-5,4		
PA3786		hypothetical protein	HUU	5,3		
PA3792	*leuA*	2-isopropylmalate synthase	ABM	29,7		-20,1
PA3795		probable oxidoreductase	PE		4,7	
PA3810	*hscA*	heat shock protein HscA	CHP		-5,0	
PA3814	*iscS*	L-cysteine desulphurase	ABM ; BC		-5,8	
PA3823	*tgt*	queuine tRNA-ribosyltransferase	TRD ; TPD		-4,8	
PA3824	*queA*	SAM:trna ribosyltransferase-isomerase	TPD		-7,1	
PA3841	*exoS*	exoenzyme S	SF	-7,7	-10,7	
PA3842		probable chaperone	SF ; CHP	-13,9	-14,3	
PA3843		hypothetical protein	HUU	-8,6	-8,6	
PA3848		hypothetical protein	HUU	3,9		
PA3871		probable peptidyl-prolyl cis-trans isomerase	TPD ; CHP	-6,7	-7,0	
PA3873	*narJ*	respiratory nitrate reductase delta chain	EM	-8,0	-8,4	
PA3874	*narH*	respiratory nitrate reductase beta chain	EM	-7,8	-7,8	
PA3876	*narK2*	nitrite extrusion protein 2	MP ; TSM	-12,6	-11,7	
PA3877	*narK1*	nitrite extrusion protein 1	MP ; TSM	-13,3	-14,0	
PA3885	*tpbA*	protein tyrosine phosphatase TpbA	MA ; TPD	-5,7	-7,0	
PA3899		probable sigma-70 factor, ECF subfamily	TR	-20,7	-16,7	
PA3910		hypothetical protein	HUU		12,7	12,7
PA3915	*moaB1*	molybdopterin biosynthetic protein B1	BC	-15,3	-15,4	
PA3922		conserved hypothetical protein	HUU	7,8	6,8	
PA3931		conserved hypothetical protein	HUU		-6,3	
PA3933		probable choline transporter	MP ; TSM	-5,2	-8,6	
PA3957		probable short-chain dehydrogenase	PE		5,9	
PA3967		hypothetical protein	HUU		-5,8	
PA3986		hypothetical protein	HUU		6,0	
PA4003	*pbpA*	penicillin-binding protein 2	CLC	-6,5	-6,4	
PA4010		hypothetical protein	HUU		8,0	
PA4063		hypothetical protein	HUU	-45,0		21,5
PA4064		probable ATP-binding component of ABC trans.	TSM	-21,3	-4,8	4,4
PA4065		hypothetical protein	MP	-10,0	-5,2	
PA4066		hypothetical protein	HUU	-8,5		

PA4073		probable aldehyde dehydrogenase	PE	4,1		
PA4091	*hpaA*	4-hydroxyphenylacetate 3-monooxygenase l. ch.	CC	24,0	10,6	
PA4092	*hpaC*	4-hydroxyphenylacetate 3-monooxygenase s. ch.	CC	24,6	20,2	
PA4107		hypothetical protein	HUU		-4,6	
PA4110	*ampC*	beta-lactamase precursor	AP	-48,3	-33,7	
PA4111		hypothetical protein	HUU	-64,5	-72,5	
PA4121		conserved hypothetical protein	HUU	10,6		
PA4122		conserved hypothetical protein	HUU	8,3		
PA4123	*hpcC*	5-carboxy-2-hydroxymuconate semialdehyde dehydrogenase	CC	27,2	9,9	
PA4124	*hpcB*	homoprotocatechuate 2,3-dioxygenase	CC	11,4		
PA4125	*hpcD*	5-carboxymethyl-2-hydroxymuconate isomerase	CC	9,3		-5,0
PA4126		probable major facilitator superfam transporter	MP ; CC	4,1		
PA4127	*hpcG*	2-oxo-hept-3-ene-1,7-dioate hydratase	CC	5,2		
PA4128		conserved hypothetical protein	PE	4,9		
PA4170		hypothetical protein	HUU			6,2
PA4181		hypothetical protein	HUU	10,2		-11,8
PA4182		hypothetical protein	HUU	18,2		-16,3
PA4198		probable AMP-binding enzyme	PE	5,6		-4,5
PA4218		probable transporter	MP ; TSM	-60,2	-5,4	11,1
PA4219		hypothetical protein	MP	-48,8	-10,7	4,6
PA4220		hypothetical protein	HUU	-263,2	-5,0	52,1
PA4221	*fptA*	Fe(III)-pyochelin o.m. receptor precursor	TSM	-41,7	-5,2	8,0
PA4222		probable ATP-binding component of ABC trans.	TSM	-4,8		
PA4223		probable ATP-binding component of ABC trans.	MP ; TSM	-23,4		5,8
PA4224	*pchG*	pyochelin biosynthetic protein PchG	TSM ; MP	-76,3		18,0
PA4225	*pchF*	pyochelin synthetase	SF ; TSM	-19,2	-4,4	4,3
PA4226	*pchE*	dihydroaeruginoic acid synthetase	SF ; TSM	-47,8		11,9
PA4227	*pchR*	transcriptional regulator PchR	TR		-13,9	5,1
PA4228	*pchD*	pyochelin biosynthesis protein PchD	SF ; TSM	-27,9		6,5
PA4229	*pchC*	pyochelin biosynthetic protein PchC	SF ; TSM	-45,7	-7,7	5,9
PA4230	*pchB*	salicylate biosynthesis protein PchB	SF ; TSM	-38,8		9,9
PA4231	*pchA*	salicylate biosynthesis isochorismate synthase	SF ; TSM	-18,4	-11,3	
PA4237	*rplQ*	50S ribosomal protein L17	TPD	-5,5	-5,4	
PA4247	*rplR*	50S ribosomal protein L18	TPD		-4,9	
PA4250	*rpsN*	30S ribosomal protein S14	TPD		-6,2	
PA4251	*rplE*	50S ribosomal protein L5	TPD		-5,1	
PA4271	*rplL*	50S ribosomal protein L7 / L12	TPD		-4,6	
PA4290		probable Chemotaxis transducer	AP ; C	10,2		
PA4296	*pprB*	two-component response regulator, PprB	TR ; TCR	15,7	-4,6	
PA4350		conserved hypothetical protein	HUU		65,4	
PA4351		probable acyltransferase	FPM		31,4	65,6
PA4352		conserved hypothetical protein	HUU	4,2		31,4
PA4357		conserved hypothetical protein	HUU	-23,4	-5,1	
PA4358		probable ferrous iron transport protein	MP ; TSM	-63,3	-5,4	4,6
PA4359		conserved hypothetical protein	HUU	-9,6		11,6
PA4364		hypothetical protein	HUU	15,8		5,1
PA4365		probable transporter	MP ; TSM	10,9		-13,9
PA4370	*icmP*	Insulin-cleaving metalloproteinase o.m. protein precursor	MP	-6,1		-8,8
PA4373		hypothetical protein	HUU	-8,2	-4,9	
PA4432	*rpsI*	30S ribosomal protein S9	TPD		-5,8	
PA4433	*rplM*	50S ribosomal protein L13	TPD		-4,5	
PA4467		hypothetical protein	MP	-38,2	-31,4	
PA4468	*sodM*	superoxide dismutase	AP	-20,2	-15,6	
PA4469		hypothetical protein	HUU	-14,1	-10,7	
PA4470	*fumC1*	fumarate hydratase	EM	-42,4	-24,7	
PA4471		hypothetical protein	HUU	-6,1	-6,1	
PA4480	*mreC*	rod shape-determining protein MreC	CLC		-4,8	
PA4507		hypothetical protein	MP	31,2	9,8	
PA4515	*piuC*	Uncharacterized iron-regulated protein	HUU	-6,8	-5,4	
PA4537		hypothetical protein	HUU		5,2	
PA4550	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	CLC		7,2	5,4
PA4551		conserved hypothetical protein	HUU			5,7
PA4552	*pilW*	type 4 fimbrial biogenesis protein PilW	MA		5,7	4,4
PA4553	*pilX*	type 4 fimbrial biogenesis protein PilX	MA		5,3	5,1
PA4554		conserved hypothetical protein	HUU			4,7
PA4555		probable permease of ABC transporter	MP ; TSM			3,6
PA4556		probable ATP-binding component of ABC trans.	TSM			3,7
PA4557	*lytB*	LytB protein	AP	-5,6	-6,5	
PA4570		HUU; weak similarity to IclR repressor family	HUU	-43,3	-5,1	8,4
PA4573		hypothetical protein	HUU		4,9	
PA4577		hypothetical protein	HUU		5,0	
PA4587	*ccpR*	cytochrome c551 peroxidase precursor	EM	7,1	6,3	
PA4602	*glyA3*	serine hydroxymethyltransferase	ABM	-5,4	-12,3	
PA4606		conserved hypothetical protein	AP	3,9		
PA4607		hypothetical protein	HUU	12,3	15,7	

PA4608		hypothetical protein	HUU	6,1	9,5	
PA4611		hypothetical protein	HUU	10,7	13,3	
PA4630		hypothetical protein	HUU	10,9	7,5	
PA4635		conserved hypothetical protein	HUU			5,6
PA4661	*pagL*	Lipid A 3-O-deacylase	HUU		7,0	4,1
PA4670	*prs*	ribose-phosphate pyrophosphokinase	CC ; NM		-5,4	
PA4671		probable ribosomal protein L25	AP ; TPD		-6,0	
PA4672		peptidyl-tRNA hydrolase	TPD		-7,7	
PA4673		conserved hypothetical protein	HUU	-6,1	-11,5	
PA4691		hypothetical protein	HUU	-9,0		
PA4692		conserved hypothetical protein	HUU	-10,8		
PA4695	*ilvH*	acetolactate synthase isozyme III small subunit	BC ; ABM			-4,0
PA4696	*ilvI*	acetolactate synthase large subunit	BC ; ABM	4,4		-6,1
PA4702		hypothetical protein	HUU	6,0	7,3	
PA4703		hypothetical protein	HUU	8,1	8,8	
PA4706		probable ATP-binding component of ABC trans.	TSM	-5,6	-7,1	
PA4707		probable permease of ABC transporter	MP ; TSM	-6,7	-9,1	
PA4708	*phuT*	Heme-transport protein, PhuT	TSM	-7,2	-8,1	
PA4709		probable hemin degrading factor	PE	-7,4	-15,7	
PA4710	*phuR*	Haem/Haemoglobin uptake o.m. receptor PhuR precursor	TSM	-9,7	-25,8	
PA4733	*acsB*	acetyl-coenzyme A synthetase	CC ; CIM	6,0	9,2	
PA4739		conserved hypothetical protein	HUU	4,6		
PA4740	*pnp*	polyribonucleotide nucleotidyltransferase	TRD		-6,0	
PA4742	*truB*	tRNA pseudouridine 55 synthase	TPD ; TRD		-6,1	
PA4743	*rbfA*	ribosome-binding factor A	TPD ; AP	-8,5	-17,6	
PA4744	*infB*	translation initiation factor IF-2	TP		-6,2	
PA4776	*pmrA*	PmrA: two-component regulator system response regulator PmrA	TCR		-6,9	
PA4787		probable transcriptional regulator	TR	7,3	8,2	
PA4803		hypothetical protein	HUU		9,2	4,5
PA4810	*fdnI*	nitrate-inducible formate dehydrogenase, gamma subunit	EM	12,9	10,0	
PA4811	*fdnH*	nitrate-inducible formate dehydrogenase, beta subunit	EM	20,4	17,1	
PA4812	*fdnG*	formate dehydrogenase-O, major subunit	EM	28,6	19,7	
PA4832		probable short-chain dehydrogenase	PE	-9,1	-7,7	
PA4834		hypothetical protein	MP	-15,0		
PA4835		hypothetical protein	HUU ; MP	-36,9	-9,2	
PA4836		hypothetical protein	HUU	-27,9	-5,2	5,4
PA4837		probable o.m. protein precursor	MP ; TSM	-43,7	-6,1	7,2
PA4838		hypothetical protein	HUU	-26,8	-5,1	5,2
PA4880		probable bacterioferritin	CIM	7,1	7,2	
PA4884		hypothetical protein	HUU	-10,2	-10,2	
PA4895		probable transmembrane sensor	MP ; TR	-6,0	-6,0	
PA4896		probable sigma-70 factor, ECF subfamily	TR	-21,5	-19,0	
PA4915		probable Chemotaxis transducer	AP ; C	6,3	6,7	
PA4925		conserved hypothetical protein	HUU	7,1	6,1	
PA4932	*rplI*	50S ribosomal protein L9	TPD	-6,0	-9,5	
PA4933		hypothetical protein	MP	-4,5		
PA4934	*rpsR*	30S ribosomal protein S18	TPD		-4,8	
PA4935	*rpsF*	30S ribosomal protein S6	TPD		-4,7	
PA5106		conserved hypothetical protein	HUU		6,4	
PA5116		probable transcriptional regulator	TR		9,7	4,3
PA5117	*typA*	regulatory protein TypA	AP	-6,9	-9,5	
PA5118	*thiI*	thiazole biosynthesis protein ThiI	BC		-11,7	
PA5153		amino acid (lysine/arginine/ornithine/histidine/ octopine) ABC transporter periplasmic binding pr.	TSM	3,8	6,3	
PA5155		amino acid (lysine/arginine/ornithine/histidine/ octopine) ABC transporter membrane protein	MP ; TSM		9,6	
PA5167		probable c4-dicarboxylate-binding protein	MP ; TSM		8,3	
PA5168		probable dicarboxylate transporter	MP ; TSM		6,5	
PA5169		probable C4-dicarboxylate transporter	MP ; TSM	4,8	16,8	
PA5173	*arcC*	carbamate kinase	ABM	-4,6		
PA5196		hypothetical protein	HUU		5,8	
PA5202		hypothetical protein	HUU		-5,0	
PA5217		probable binding protein component of ABC iron .	TSM	-5,8		5,1
PA5233		hypothetical protein	HUU	4,2		
PA5288	*glnK*	nitrogen regulatory protein P-II 2	CIM	4,6		
PA5315	*rpmG*	50S ribosomal protein L33	TPD		-8,9	
PA5348		probable DNA-binding protein	DRR	9,3	15,9	
PA5351	*rubA1*	Rubredoxin 1	CC		4,9	
PA5359		hypothetical protein	HUU		6,3	
PA5360	*phoB*	two-component response regulator PhoB	TR ; TCR		38,4	41,4
PA5365	*phoU*	phosphate uptake regulatory protein PhoU	MP ; TR		7,3	14,2
PA5366	*pstB*	ATP-binding component of ABC phosphate transporter	MP ; TSM		15,4	33,5
PA5367	*pstA*	membrane protein component of ABC phosphate	MP ; TSM		12,6	16,7

		transporter				
PA5368	*pstC*	membrane protein component of ABC phosphate transporter	MP ; TSM		7,4	7,4
PA5369	*pstS*	phosphate ABC transporter, periplasmic phosphate-binding protein, PstS	TSM		16,5	22,3
PA5372	*betA*	choline dehydrogenase	ABM ; AP	-6,8	-12,4	
PA5373	*betB*	betaine aldehyde dehydrogenase	ABM ; AP	-10,3	-25,4	
PA5374	*betI*	transcriptional regulator BetI	TR	-9,5	-33,0	
PA5380	*gbdR*	GbdR	TR	4,9		
PA5396		hypothetical protein	HUU	8,1		
PA5410	*gbcA*	GbcA	ABM	8,3		
PA5415	*glyA1*	serine hydroxymethyltransferase	ABM		4,8	4,3
PA5424		conserved hypothetical protein	MP		4,9	
PA5429	*aspA*	aspartate ammonia-lyase	ABM		4,9	13,3
PA5436		probable biotin carboxylase subunit of a transcarboxylase	CIM	3,9		
PA5445		probable coenzyme A transferase	PE	9,9	-5,1	
PA5470		probable peptide chain release factor	TPD	-5,0	-4,9	
PA5481		hypothetical protein	HUU	10,9		
PA5482		hypothetical protein	MP	11,4	8,3	
PA5500	*znuC*	zinc transport protein ZnuC	TSM	-6,5		
PA5503		probable ATP-binding component of ABC trans.	TSM		-5,0	
PA5504		D-methionine ABC transporter membrane protein	MP ; TSM		-5,8	
PA5531	*tonB*	TonB protein	TSM	-5,5		6,2
PA5532		hypothetical protein	HUU	-19,8	-5,6	
PA5534		hypothetical protein	HUU	-14,4	-5,2	
PA5535		conserved hypothetical protein	HUU	-54,6		16,6
PA5536		conserved hypothetical protein	HUU	-46,7		13,8
PA5538	*amiA*	N-acetylmuramoyl-L-alanine amidase	CLC	-18,2		8,7
PA5539		hypothetical protein	HUU	-24,3		5,5
PA5540		hypothetical protein	HUU	-51,5	-5,1	10,2
PA5541	*pyrQ*	dihydroorotase	NM	-47,2	-5,5	8,6
PA5546		conserved hypothetical protein	PE		4,8	
Pae_flgK			MA		-6,2	
M57501cd	*flaA*	Pseudomonas aeruginosa flagellin (flaA) gene	MA	13,6	5,7	
tRNA-Cys		PA2581.1	NR	24,7	327,9	13,3
tRNA-Pro		PA2736.1/PA3031.1/ PA4541.2	NR	6,1	27,9	
tRNA-Val		PA2775.1/PA3094.3/PA3262.2	NR	4,2		
tRNA-Ser		PA2852.1/PA0905.1/ PA1013.1/PA2603.1	NR	212,9	416,9	
tRNA-Pro		PA3031.1	NR			4,6
tRNA-Asn		PA3139.1/PA4541.3	NR		5,3	
tRNA-Arg		PA3368.1/PA4581.1/PA0905.2/PA0905.3/ PA1796.1/ PA0263.1	NR		7,8	
tRNA-Leu		PA3824.1 /PA4937.1/ PA4937.2/ PA1796.3/ PA2570.1/ PA4746.2	NR	23,2	59,4	
tRNA-Thr		PA4277.1/PA4524.1/ PA5160.1	NR		46,5	12,3
tRNA-Tyr		PA4277.3	NR		5,1	4,2
tRNA-Met		PA4673.1	NR			25,5
tRNA-Met		PA4746.1/PA4673.1/ PA0574.1/PA0922.1	NR		27,9	
tRNA-Phe		PA5149.1	NR	7,3	53,6	7,4

[a] Gene ID, gene name, function and PCFC are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

[b] Abbreviations used for PseudoCAP Function Class (PCFC) are as following Adaptation, Protection (AP); Amino acid biosynthesis and metabolism (ABM); Antibiotic resistance and susceptibility (ARS); Biosynthesis of cofactors, prosthetic groups and carriers (BC);Carbon compound catabolism (CC); Cell wall, LPS, capsule (CLC); Central intermediary metabolism(CIM); Chaperones & heat shock proteins (CHP); Chemotaxis (C); DNA replication, recombination, modification and repair (DRR); Energy metabolism (EM); Fatty acid and phospholipid metabolism (FPM); Hypothetical, unclassified, unknown (HUU); Membrane proteins (MP); Motility & Attachment (MA); Non-coding RNA gene (NR); Nucleotide biosynthesis and metabolism (NM); Protein secretion/export apparatus (PSE); Putative enzymes (PE); Related to phage, transposon, or plasmid (RPT); Secreted Factors (toxins, enzymes, alginate) (SF); Transcriptional regulators (TR); Transcription, RNA processing and degradation (TRD); Translation, post-translational modification, degradation (TPD); Transport of small molecules (TSM); Two-component regulatory systems (TCR) and Unclassified genome space (UGS).

[+] Other abbreviations used are protein synthase (p.s.), outer membrane (o.m.), precursor (prc.), secretion (secr.), transporter (trans.)

7.4 Appendix B

Table 31: Summary of phenotypic characterisation of methionine auxotrophic clinical *P. aeruginosa* CF isolates

Patient	Colonizing strain	Clone[A]	Clone type[A]	Mutator pheno-type[B*]	BHL[C*] [µM] / OD_{578nm}	odDHL[D*] [nM] / OD_{578nm}	Swimming[E*] [mm]	Swarming[F*] [mm]	Twitching[G*] [mm]
Patient 1	**KI-AW 1**	P	2C2A	**1,09E-07 ± 8,80E-08**	**28,9 ± 2,5**	**26,0 ± 2,0**	5,3 ± 0,6	6,0 ± 1,0	3,0 ± 1,0
Patient 4	KI-AW 10	I	F429	4,64E-07 ± 5,58E-07	18,3 ± 5,9	**77,9 ± 3,5**	**14,7 ± 0,6**	4,5 ± 0,5	2,2 ± 0,8
Patient 7	KI-AW 16		3BAA	7,15E-07 ± 4,26E-07	15,3 ± 7,1	1,7 ± 0,9	3,3 ± 0,6	1,8 ± 0,3	8,7 ± 0,6
Patient 8	KI-AW 23		441A	7,23E-07 ± 2,44E-07	23,2 ± 12,1	2,2 ± 0,3	4,7 ± 1,5	5,3 ± 2,1	5,3 ± 0,6
Patient 9	KI-AW 20	M	6E12	2,80E-08 ± 3,00E-08	**38,2 ± 4,1**	**70,8 ± 4,4**	2,2 ± 0,3	3,0 ± 1,0	3,3 ± 0,6
Patient 11	KI-AW 22		2D92	4,33E-08 ± 3,65E-08	18,0 ± 6,4	3,2 ± 0,2	**21,0 ± 6,6**	**10,7 ± 0,6**	**8,3 ± 3,8**
Patient 13	KI-AW 25		6862	2,36E-08 ± 1,76E-08	**48,3 ± 13,1**	3,2 ± 1,6	1,7 ± 0,6	2,3 ± 0,6	3,3 ± 0,6
Patient A	CFCZ		E59A	3,16E-08 ± 4,04E-08	21,2 ± 10,1	2,2 ± 0,6	5,0 ± 1,0	2,7 ± 0,6	3,0 ± 1,0
Patient 3	KI-AW 4	P	6C2A	8,76E-08 ± 1,25E-07	13,1 ± 10,0	0,9 ± 0,4	**7,0 ± 0,1**	5,3 ± 0,6	3,0 ± 1,0
	KI-AW 5	P	6C2A	2,97E-08 ± 2,67E-08	13,1 ± 7,4	4,5 ± 3,5	**13,7 ± 0,6**	6,0 ± 1,0	3,2 ± 0,8
	KI-AW 7	P	6C2A	1,54E-08 ± 1,05E-08	27,9 ± 13,8	6,0 ± 2,5	**12,0 ± 1,0**	3,3 ± 0,6	2,8 ± 1,3
	KI-AW 8	P	6C2A	1,67E-08 ± 1,08E-08	26,2 ± 16,8	0,3 ± 0,4	**13,7 ± 0,6**	**7,3 ± 1,5**	3,3 ± 0,6
	KI-AW 9	P	6C2A	1,45E-07 ± 2,08E-07	7,1 ± 2,0	1,1 ± 1,0	**11,0 ± 2,0**	**8,3 ± 1,5**	3,3 ± 0,6
	KI-AW 21	P	6C2A	2,33E-08 ± 2,88E-08	28,0 ± 20,1	7,2 ± 4,4	**12,7 ± 1,5**	5,3 ± 0,6	3,7 ± 0,6
	KI-AW 6	P	6C2A	**8,20E-06 ± 9,05E-06**	20,9 ± 9,2	**46,6 ± 1,9**	3,0 ± 1,0	1,8 ± 0,3	3,2 ± 0,8
Patient 2	**KI-AW 2**	B	E429	**1,31E-06 ±1,00E-06**	5,7 ± 5,0	3,3 ± 3,9	4,3 ± 0,6	1,5 ± 0,5	**8,3 ± 1,5**
	KI-AW 3	B	E429	**1,57E-06 ±1,77E-06**	16,7 ± 6,3	4,4 ± 4,2	3,7 ± 0,6	0,5 ± 0,1	6,7 ± 1,5
Patient 4	**KI-AW 11**	I	F429	**2,77E-06 ±4,53E-06**	**32,3 ± 3,2**	7,0 ± 2,3	**13,0 ± 1,0**	**8,3 ± 0,6**	1,7 ± 0,3
Patient 5	**KI-AW 12**	I	F429	**1,92E-06 ±1,64E-06**	11,2 ± 5,7	1,9 ± 1,3	1,5 ± 0,5	3,3 ± 0,6	3,8 ± 0,3
Patient 6	**KI-AW 14**	E	1BAE	**2,67E-06 ±8,04E-07**	6,8 ± 3,1	3,7 ± 1,9	3,7 ± 0,6	6,3 ± 1,5	5,7 ± 1,2
	KI-AW 15	E	1BAE	**8,08E-06 ±1,13E-05**	10,3 ± 6,7	2,4 ± 2,0	3,3 ± 0,6	**8,0 ± 2,0**	4,7 ± 0,6
	KI-AW 26	E	1BAE	**5,09E-06 ± 2,03E-06**	18,0 ± 11,1	2,3 ± 0,4	2,3 ± 1,2	4,3 ± 0,6	5,3 ± 0,6
	KI-AW 27	E	1BAE	**1,52E-06 ± 4,87E-07**	12,9 ± 8,6	2,4 ± 0,5	4,0 ± 1,0	5,0 ± 1,0	4,7 ± 0,6
Patient 8	**KI-AW 17**		441A	**1,67E-05 ±1,23E-05**	6,4 ± 3,2	2,5 ± 1,8	3,7 ± 1,5	5,3 ± 2,5	5,7 ± 0,6
	KI-AW 18		441A	**3,71E-06 ±4,31E-06**	18,4 ± 5,9	1,9 ± 0,9	4,0 ± 1,0	4,0 ± 1,0	4,3 ± 0,6
	KI-AW 19		441A	**1,83E-05 ±2,86E-05**	11,6 ± 9,9	0,3 ± 0,3	2,7 ± 0,6	1,8 ± 0,3	4,3 ± 0,6
	KI-AW 24		441A	**1,77E-06 ±1,46E-06**	20,0 ± 7,1	2,1 ± 0,2	1,7 ± 0,6	3,3 ± 0,6	4,3 ± 0,6
Patient 12	**KI-AW 28**		2F82	**2,61E-06 ±4,06E-06**	**32,9 ± 4,1**	2,2 ± 0,2	1,8 ± 1,3	2,3 ± 0,6	3,7 ± 0,6
	PAO1 Δ*mutS*			**4,94E-006 ±2,76E-06**	ND	ND	ND	ND	ND
	PAO1 Δ*metF*			7,16E-08 ± 1,03E-07	**45,1 ± 9,9**	**188,9 ± 26,9**	**24,7 ± 0,6**	**19,7 ± 6,0**	6,3 ± 1,5
	PAO1			1,54E-08 ± 2,05E-09	**48,3 ± 16,4**	**147,3 ± 3,9**	**25,3 ± 1,5**	**19,7 ± 4,5**	**8,0 ± 1,0**

[A] Clone and clone type according to single-nucleotide polymorphisms (SNPs) at seven conserved loci and two multiallelic loci (Wiehlmann *et al.*, 2007)

[B] Mutator phenotype was determined as CFU of rifampicin 100 µg/ml (RA-100) resistant bacteria vs. CFU. Strains with a mutator frequency ≥ 1.5 x 10-7 were classified as strong mutators (bold black). Strains with a mutator frequency < 1.5 x 10-7 to ≥ 1.5 x 10-8 were classified as weak mutators (bold grey). Strains with mutator frequency of < 1.5 x 10-8 were classified as non-mutator. Experiments were performed by Matthias Rottmann (Rottmann, 2011)

[C] BHL [µM] / OD_{578} was determined. Results above the average > 20 ± 8 are marked in bold letters.

[D] odDHL [nM] / OD_{578} was determined. Results above the average > 10 ± 3 are marked in bold letters.

[E] Swimming as the radius [mm] of the respective swimming halo was determined. Results above the average radius of > 6.5 ± 1 mm are marked in bold letters.

[F] Swarming as the radius [mm] of the respective swarming halo was determined. Results above the average radius of > 4.5 ± 1 mm are marked in bold letters.

[G] Twitching as the radius [mm] of the respective twitching halo was determined. Results above the average radius of > 4.5 ± 1 mm are marked in bold letters.

***** Results of at least three independent experiments are shown. The standard deviation of the mean is shown.

Table 32: Mutations in *metF* (PA0430) nucleotide sequence (detailed single base exchange)

Patient	Colonizing strain[C]	Mutations unique for isolate	481222[A] (P 25)	481225[A] (P 26)	481229[A] (P 33)	481241[A] (P 45)	481312[A] (P116)	481386[A] (P 190)
Patient 1	**KI-AW 1**	481734 C>T 482015 T>C						T>C
Patient 2	**KI-AW 2**				T I*	G>A		T>C
	KI-AW 3	481987 no seq.*			T I*	G>A		T>C
Patient 3	KI-AW 4	481201 T>G 481203 I* (34 bp) 481205 G >A 481206 G>A 481196 T>A	A >C	T>C				T>C
	KI-AW 5							T>C
	KI-AW 6		A >C					T>C
	KI-AW 7			T>C				T>C
	KI-AW 8		A >C					T>C
	KI-AW 9							T>C
	KI-AW 21							T>C
Patient 4	**KI-AW 10**	482010 T>C						
	KI-AW 11	482010 T>C						
Patient 5	**KI-AW 12**	481432 C >D*						
Patient 6	**KI-AW 14**							
	KI-AW 15		A >C	T>C				
	KI-AW 26							
	KI-AW 27							
Patient 7	**KI-AW 16**	481356 G>A						
Patient 8	**KI-AW 17**						C> D*	
	KI-AW 18						C> D*	
	KI-AW 19	481225 G>T	A >C	T>C			C> D*	
	KI-AW 23						C> D*	
	KI-AW 24						C> D*	
Patient 9	KI-AW 20	481269 G>A 481623 A I*						
Patient 10	KI-AW 22	481608 – 481617 D*						
Patient 11	KI-AW 25	481436 T>C						
Patient 12	KI-AW 28	481659 C I* 481667 C>T						
Patient A	CFCZ							
Patient C	65	481525 G>A						
Patient D	BT72	481807 T>A						

[A] Genomic position in *P. aeruginosa* PAO1 genome, and base position Pxxx in the *metF* gene (873 bp)

[C] Strains with a mutator frequency ≥ 1.5 x 10-7 were classified as strong mutators (bold black). Strains with a mutator frequency < 1.5 x 10-7 to ≥ 1.5 x 10-8 were classified as weak mutators (bold grey). Strains with mutator frequency of < 1.5 x 10-8 were classified as non-mutator.

I* insertion

D* deletion

no seq.* no sequence afterwards

Table 32: Continued mutations in *metF* (PA0430) nucleotide sequence (detailed single base exchange)

Patient	Colonizing strain[C]	481416[A] (P 220)	481431[A] (P 235)	481454[A] (P 258)	481491[A] (P 295)	481500[A] (P 304)	481550[A] – 481562 (P 354 - 366)	481648[A] (P 452)	482051[A] (P 855)
Patient 1	**KI-AW 1**		G>C					G>A	
Patient 2	**KI-AW 2**					C>T		G>A	
	KI-AW 3					C>T		G>A	
Patient 3	KI-AW 4				C>A	C>T	D*	G>A	
	KI-AW 5				C>A	C>T	D*	G>A	
	KI-AW 6				C>A	C>T	D*	G>A	
	KI-AW 7				C>A	C>T	D*	G>A	
	KI-AW 8				C>A	C>T	D*	G>A	
	KI-AW 9				C>A	C>T	D*	G>A	
	KI-AW 21				C>A	C>T	D*	G>A	
Patient 4	**KI-AW 10**		G>C					G>A	
	KI-AW 11	G>A	G>C					G>A	
Patient 5	**KI-AW 12**	G>A	G>C					G>A	
Patient 6	**KI-AW 14**	G>A	G>C	C>A					
	KI-AW 15		G>C	C>A					
	KI-AW 26		G>C	C>A					
	KI-AW 27		G>C	C>A					
Patient 7	**KI-AW 16**		G>C						A I*
Patient 8	**KI-AW 17**								A I*
	KI-AW 18								
	KI-AW 19								
	KI-AW 23								
	KI-AW 24								
Patient 9	KI-AW 20								
Patient 10	KI-AW 22								
Patient 11	KI-AW 25								
Patient 12	KI-AW 28								
Patient A	CFCZ		G>C					G>A	
Patient C	65	G>A	G>C					G>A	
Patient D	BT72	G>A	G>C					G>A	

[A] Genomic position in *P. aeruginosa* PAO1 genome, and base position Pxxx in the *metF* gene (873 bp)

[C] Strains with a mutator frequency ≥ 1.5 x 10-7 were classified as strong mutators (bold black). Strains with a mutator frequency < 1.5 x 10-7 to ≥ 1.5 x 10-8 were classified as weak mutators (bold grey). Strains with mutator frequency of < 1.5 x 10-8 were classified as non-mutator.

I* insertion

D* deletion

no seq.* no sequence afterwards

Table 33: Amino acid exchanges in MetF (PA0430) protein sequence resulting from nucleotide sequence (detailed amino acid exchange)

Patient	Colonizing strain[C]	Length of amino acid seq. [aa]	Amino acid exchanges for each strain (unique to db* event) *	Amino acid M9	Amino acid G 28	Amino acid L 62	Amino acid S 86
Patient 1	**KI-AW 1**	178	**Q 179 SC***				
Patient 2	**KI-AW 2**	159	Q 11 H; FS* **D 160 SC***				
	KI-AW 3	29	Q 11 H; FS* **E 30 SC***				
Patient 3	KI-AW 4	40	V 2 G; FS* **G 41 SC***				
	KI-AW 5	163					
	KI-AW 6	163		M 9 L			
	KI-AW 7	163		M 9 T			
	KI-AW 8	163		M 9 L			
	KI-AW 9	163					
	KI-AW 21	163					
Patient 4	**KI-AW 10**	290	Y 271 H				
	KI-AW 11	290	Y 271 H				
Patient 5	**KI-AW 12**	168	H 79 T;FS* S 81 P **V 169 SC***				
Patient 6	**KI-AW 14**	85					**S 86 SC***
	KI-AW 15	85		M 9 P			**S 86 SC***
	KI-AW 26	85					**S 86 SC***
	KI-AW 27	85					**S 86 SC***
Patient 7	**KI-AW 16**	284	G 54 S **N 285 SC***				
Patient 8	**KI-AW 17**	61			G 28 A; FS*	**L 62 SC***	
	KI-AW 18	61			G 28 A; FS*	**L 62 SC***	
	KI-AW 19	61	S 10 I	M 9 P	G 28 A; FS*	**L 62 SC***	
	KI-AW 23	61			G 28 A; FS*	**L 62 SC***	
	KI-AW 24	61			G 28 A; FS*	**L 62 SC***	
Patient 9	KI-AW 20	160	H 142 Q **D 161 SC***				
Patient 10	KI-AW 22	164	G138 S; FS* **V 165 SC***				
Patient 11	KI-AW 25	290	L 80 P				
Patient 12	KI-AW 28	156	Q 154 P A 155 G R 156 A **S 157 SC***				
Patient A	CFCZ	290					
Patient C	65	290	G 110 S				
Patient D	BT72	290	V 203 E				

FS* frame shift after the indicated amino acid exchange

SC* stop codon. Insertion of stop codon instead of amino acid is marked in bold letters.

db* duplicate

* Amino acid exchanges are unique and or in maximale in second strain occurring for clarity reasons not listed as as a single event in the table.

[C] Strains with a mutator frequency ≥ 1.5 x 10-7 were classified as strong mutators (bold black). Strains with a mutator frequency < 1.5 x 10-7 to ≥ 1.5 x 10-8 were classified as weak mutators (bold grey). Strains with mutator frequency of < 1.5 x 10-8 were classified as non-mutator.

Table 33: Continued amino acid exchanges in MetF (PA0430) protein sequence resulting from nucleotide sequence (detailed amino acid exchange)

Patient	Colonizing strain[C]	Length of amino acid seq. [aa]	Amino acid M 118	Amino acid F 164
Patient 1	**KI-AW 1**	178		
Patient 2	**KI-AW 2**	159		
	KI-AW 3	29		
Patient 3	KI-AW 4	40		
	KI-AW 5	163	M 118 N; FS*	**F 164 SC***
	KI-AW 6	163	M 118 N; FS*	**F 164 SC***
	KI-AW 7	163	M 118 N; FS*	**F 164 SC***
	KI-AW 8	163	M 118 N; FS*	**F 164 SC***
	KI-AW 9	163	M 118 N; FS*	**F 164 SC***
	KI-AW 21	163	M 118 N; FS*	**F 164 SC***
Patient 4	**KI-AW 10**	290		
	KI-AW 11	290		
Patient 5	**KI-AW 12**	168		
Patient 6	**KI-AW 14**	85		
	KI-AW 15	85		
	KI-AW 26	85		
	KI-AW 27	85		
Patient 7	**KI-AW 16**	284		
Patient 8	**KI-AW 17**	61		
	KI-AW 18	61		
	KI-AW 19	61		
	KI-AW 23	61		
	KI-AW 24	61		
Patient 9	KI-AW 20	160		
Patient 10	KI-AW 22	164		
Patient 11	KI-AW 25	290		
Patient 12	KI-AW 28	156		
Patient A	CFCZ	290		
Patient C	65	290		
Patient D	BT72	290		

FS* frame shift after the indicated amino acid exchange

SC* stop codon. Insertion of stop codon instead of amino acid is marked in bold letters.

db* duplicate

* Amino acid exchanges are unique and or in maximal occurring in a second strain, for clarity reasons these exchanges are not listed as a single event in the table.

[C] Mutator phenotype in colonizing strains is shown in bold letters.

Table 34: Microarray analysis of regulated genes in PAO1 Δ*metF* compared to PAO1 in M9 0.5 % caseinate after growth for 4 h and 12 h

Differentially regulated genes of *P. aeruginosa* PAO1 compared to PAO1 Δ*metF* after 4 h and after 12 h in M9 0.5 % caseinate under microaerobic to anaerobic conditions. Shown are the gene ID, gene name, function and the fold change (FC) of genes differently regulated of PAO1 wt 4 h vs. PAO1 Δ*metF* 4 h (W4 / M4), PAO1 wt 12 h vs. PAO1 Δ*metF* wt 12 h (W12 / M12), PAO1 Δ*metF* 12 h vs PAO1 Δ*metF* 4 h (M12 / M4) and PAO1 wt 12 h vs PAO1 wt 4 h (W12 / W4).

Gene ID[a]	Gene name[a]	Function[a]	PCFC[a,b]	FC W4/ M4	W12/ M12	M12/ M4	W12/ W4
1427453 -1428080		Unknown function	UGS		-2,0	3,7	2,7
1996806 -1997509		Unknown function	UGS				-2,6
2568929 -2568284		Unknown function	UGS			2,5	
2722174 -2721530		Unknown function	UGS		-2,1	4,4	
3527428 -3526677		Unknown function	UGS		-2,2	2,1	
3649704 -3648915		Unknown function	UGS			3,0	
4713098 -4713795		Unknown function	UGS			15,1	11,5
517462 - 518083		Unknown function	UGS		-2,3		-2,0
545644 - 546334		Unknown function	UGS			2,3	
5563286 -5563964		Unknown function	UGS			-2,0	-2,3
629884 - 630527		Unknown function	UGS		-2,0	2,2	
721556 - 727608		Unknown function	UGS	-2,4			2,2
991198 – 991830		Unknown function	UGS	-2,0			
PA0001	*dnaA*	chromosomal replication initiator protein DnaA	DRR			-2,3	
PA0002	*dnaN*	DNA polymerase III, beta chain	DRR			-2,4	
PA0009	*glyQ*	glycyl-tRNA synthetase alpha chain	ABM			-2,1	
PA0020		hypothetical protein	HUU			-2,8	
PA0026	*plcB*	phospholipase C, PlcB	SF				-2,0
PA0036	*trpB*	tryptophan synthase beta chain	ABM			-2,1	
PA0038		hypothetical protein	HUU				-2,4
PA0040		conserved hypothetical protein	HUU	-2,3			
PA0048		probable transcriptional regulator	TR	2,4			
PA0048		probable transcriptional regulator	TR			-4,0	-11,0
PA0049		hypothetical protein	HUU	2,5		-4,2	-12,8
PA0050		hypothetical protein	HUU		-3,7		
PA0052		hypothetical protein	HUU	2,5			-4,8
PA0055		hypothetical protein	HUU		2,4	-5,1	-3,2
PA0059	*osmC*	osmotically inducible protein OsmC	Ap	2,3			-2,4
PA0070		hypothetical protein	MP			-4,0	-2,2
PA0075	*pppA*	PppA	PE ; PSE			-2,3	
PA0084		conserved hypothetical protein	HUU			-2,2	
PA0085	*hcp1*	Hcp1	SF			-2,7	
PA0094		hypothetical protein	HUU			-2,6	
PA0105	*coxB*	cytochrome c oxidase, subunit II	EM	2,5		-3,4	-4,9
PA0106	*coxA*	cytochrome c oxidase, subunit I	EM	3,3	3,5	-7,2	-6,9
PA0107		conserved hypothetical protein	EM	3,6	3,7	-8,6	-8,3
PA0108	*coIII*	cytochrome c oxidase, subunit III	EM	3,1	3,5	-6,4	-5,7
PA0110		hypothetical protein	HUU	2,4	2,2	-3,2	-3,4
PA0111		hypothetical protein	HUU	2,9	3,2	-5,9	-5,3
PA0112		hypothetical protein	MP	3,0	2,4	-4,7	-6,1
PA0113		probable cytochrome c oxidase assembly fc.*	EM	3,5	2,7	-5,2	-6,7
PA0114	*senC*	SenC	HUU	3,2		-3,5	-6,7
PA0115		conserved hypothetical protein	HUU	-2,0			
PA0118		hypothetical protein	PE			-3,8	-3,7
PA0122		conserved hypothetical protein	HUU			9,3	5,5
PA0132		beta-alanine--pyruvate transaminase	ABM				-3,2
PA0139	*ahpC*	alkyl hydroperoxide reductase subunit C	Ap			-2,7	-2,1
PA0141		conserved hypothetical protein	HUU		-2,6	3,9	
PA0161		hypothetical protein	HUU			5,1	3,3
PA0167		probable transcriptional regulator	TR			-2,5	

PA0169		hypothetical protein	HUU			-2,0	
PA0170		hypothetical protein	HUU			-2,9	
PA0171		hypothetical protein	MA			-2,5	
PA0172		hypothetical protein	HUU	-2,0			
PA0173		probable methylesterase	Ap ;C	2,7			-3,6
PA0174		conserved hypothetical protein	HUU	2,9			-3,3
PA0175		probable Cprotein methyltransferase	TR ; Ap	3,3			-4,1
PA0176	*aer2*	aerotaxis transducer Aer2	Ap ;C	2,6			-3,7
PA0177		probable purine-binding Cprotein	Ap ;C	2,1			-2,9
PA0178		probable two-component sensor	C; Ap	2,1			-2,9
PA0179		probable two-component response regulator	C; Ap	2,7			-3,8
PA0180		probable Ctransducer	Ap ;C	2,4			-3,6
PA0200		hypothetical protein	HUU		-3,3	16,9	3,3
PA0201		hypothetical protein	HUU			2,1	
PA0208	*mdcA*	malonate decarboxylase alpha subunit	CC		2,5		2,6
PA0209		conserved hypothetical protein	PE			2,2	2,8
PA0210	*mdcC*	malonate decarboxylase delta subunit	CC		2,3		3,7
PA0211	*mdcD*	malonate decarboxylase beta subunit	CC				2,2
PA0212	*mdcE*	malonate decarboxylase gamma subunit	CC			2,4	3,5
PA0213		hypothetical protein	CC			4,1	5,6
PA0214		probable acyl transferase	CC		2,1	2,4	4,5
PA0215		malonate transporter MadL	MP			2,4	3,1
PA0216		malonate transporter MadM	MP		2,4	2,2	3,1
PA0250		conserved hypothetical protein	HUU				-2,4
PA0256		hypothetical protein	HUU	2,1			-2,4
PA0263	*hcpC*	secreted protein Hcp	SF				-2,6
PA0265	*gabD*	succinate-semialdehyde dehydrogenase	CIM ; CC				-2,2
PA0266	*gabT*	4-aminobutyrate aminotransferase	CIM ; CC				-2,3
PA0274		hypothetical protein	HUU				-3,0
PA0276		hypothetical protein	MP			2,3	
PA0284		hypothetical protein	HUU				2,1
PA0296	*spuI*	probable glutamine synthetase	PE			-3,3	-3,8
PA0297	*spuA*	probable glutamine amidotransferase	ABM			-2,9	-3,9
PA0298	*spuB*	probable glutamine synthetase	PE			-4,3	-5,4
PA0299	*spuC*	putrescine aminotransferase	PE			-2,6	-4,0
PA0300	*spuD*	polyamine transport protein	TSM	2,4		-4,0	-5,4
PA0301	*spuE*	polyamine transport protein	TSM	2,0		-2,2	-3,5
PA0302	*spuF*	polyamine transport protein PotG	TSM			-2,1	-3,2
PA0303	*spuG*	polyamine transport protein PotH	MP; TSM			-2,1	-2,9
PA0311		hypothetical protein	HUU			-3,1	-2,6
PA0314		L-cysteine transporter of ABC system FliY	TSM	2,4		-2,1	-4,5
PA0315		hypothetical protein	HUU		2,5	-3,2	-2,0
PA0316	*serA*	D-3-phosphoglycerate dehydrogenase	ABM	-15,5	-4,2	-2,2	
PA0323		probable binding protein com.* of ABC tr.*	TSM				-2,8
PA0326		probable ATP-binding com.* of ABC tr.*	TSM				-2,3
PA0328		hypothetical protein	HUU			-2,5	
PA0328		hypothetical protein	HUU				-2,6
PA0329		conserved hypothetical protein	HUU	2,1			-5,3
PA0341	*lgt*	prolipoprotein diacylglyceryl transferase	TPD			-2,1	
PA0346		hypothetical protein	HUU			2,6	
PA0349		hypothetical protein	MP			-2,2	-2,5
PA0353	*ilvD*	dihydroxy-acid dehydratase	BC; ABM			-2,1	
PA0363	*coaD*	phosphopantetheine adenylyltransferase	CIM		2,6	-3,1	
PA0364		probable oxidoreductase	PE			-2,2	-3,1
PA0365		hypothetical protein	MP			-2,0	-2,9
PA0366		probable aldehyde dehydrogenase	PE				-2,8
PA0376	*rpoH*	sigma factor RpoH	TR				2,2
PA0380		conserved hypothetical protein	HUU		2,1	-2,2	
PA0382	*micA*	DNA mismatch repair protein MicA	DRR	-2,2			2,2
PA0384		hypothetical protein	HUU	2,1		-2,0	-3,2
PA0385		hypothetical protein	HUU		2,3	-2,6	
PA0388		hypothetical protein	HUU			-2,1	
PA0390	*metX*	homoserine O-acetyltransferase	ABM			-2,7	
PA0409	*pilH*	twitching motility protein PilH	TCR ; C			-2,3	
PA0430	*metF*	5,10-methylenetetrahydrofolate reductase	ABM	2,5	7,2		3,2
PA0431		hypothetical protein	HUU	-2,7			
PA0432	*sahH*	S-adenosyl-L-homocysteine hydrolase	ABM	-5,5			2,6
PA0433		hypothetical protein	HUU	-2,1			
PA0446		conserved hypothetical protein	HUU	2,1		-2,4	-4,8
PA0447	*gcdH*	glutaryl-CoA dehydrogenase	FPM; CC			-2,9	-3,6
PA0449		hypothetical protein	HUU			-2,9	-3,1
PA0456		probable cold-shock protein	TR ; Ap				2,0
PA0459		probable ClpA/B protease ATP binding sub.*	TPD		-2,5	2,1	
PA0460		hypothetical protein	HUU	2,3	-2,2		-2,7
PA0462		hypothetical protein	HUU	2,2			-4,3
PA0467		conserved hypothetical protein	HUU				-2,2
PA0468		hypothetical protein	HUU				-2,1
PA0471		probable transmembrane sensor	TCR; TR			2,1	

PA0472		probable sigma-70 factor, ECF subfamily	TR			2,6	3,5
PA0473		probable glutathione S-transferase	PE		2,3	-3,5	
PA0478		probable N-acetyltransferase	PE	-2,1			
PA0479		probable transcriptional regulator	TR				-3,8
PA0483		probable acetyltransferase	PE				-3,0
PA0484		conserved hypothetical protein	HUU	2,3			-3,2
PA0485		conserved hypothetical protein	MP			-2,8	-2,7
PA0492		conserved hypothetical protein	HUU				-3,6
PA0496		conserved hypothetical protein	HUU				-3,0
PA0500	*bioB*	biotin synthase	BC			2,2	
PA0506		probable acyl-CoA dehydrogenase	PE			-3,2	-2,3
PA0507		probable acyl-CoA dehydrogenase	PE			-4,2	-2,4
PA0508		probable acyl-CoA dehydrogenase	PE	-3,6		-4,9	
PA0509	*nirN*	probable c-type cytochrome	BC ; EM		-2,3	2,4	
PA0510		probable uroporphyrin-III c-methyltransferase	BC ; EM		-2,4	2,4	
PA0515		probable transcriptional regulator	BC ; TR		-3,0	3,6	
PA0526		hypothetical protein	HUU		-2,4	4,4	2,4
PA0540		hypothetical protein	HUU			-3,0	-3,2
PA0545		hypothetical protein	PE			2,0	
PA0546	*metK*	methionine adenosyltransferase	ABM	-5,4	-2,4		2,0
PA0547		probable transcriptional regulator	TR	-8,6	-3,2		4,1
PA0555	*fda*	fructose-1,6-bisphosphate aldolase	CIM; CC			-2,1	
PA0559		conserved hypothetical protein	HUU				2,1
PA0563		conserved hypothetical protein	HUU			-3,1	
PA0565		conserved hypothetical protein	HUU			-4,2	-5,6
PA0567		conserved hypothetical protein	MP				-3,3
PA0578		conserved hypothetical protein	HUU		3,3	-2,5	2,4
PA0579	*rpsU*	30S ribosomal protein S21	TPD	-2,5	2,7	-2,4	2,7
PA0580	*gcp*	O-sialoglycoprotein endopeptidase	TPD				2,0
PA0582	*folB*	dihydroneopterin aldolase	BC	-10,1		-3,6	
PA0583		hypothetical protein	BC	-7,9		-3,6	
PA0585		hypothetical protein	HUU	3,6			-4,4
PA0586		conserved hypothetical protein	HUU	3,5		-2,6	-11,5
PA0587		conserved hypothetical protein	HUU	3,3			-7,3
PA0588		conserved hypothetical protein	HUU	2,7		-3,0	-10,8
PA0602		probable binding protein com.of ABC tr.*	TSM			-2,6	
PA0603		probable ATP-binding com.* of ABC tr.*	TSM			-3,0	-2,3
PA0604		probable binding protein com.*of ABC tr.*	TSM			-4,9	-4,2
PA0605		probable permease of ABC transporter	MP; TSM				-2,2
PA0606		probable permease of ABC tr.*	MP; TSM			-2,6	-2,3
PA0612	*ptrB*	repressor, PtrB	TR			3,8	
PA0614		hypothetical protein	HUU			3,5	2,9
PA0615		hypothetical protein	HUU		-2,0	3,4	2,5
PA0616		hypothetical protein	RPT			3,5	2,1
PA0617		probable bacteriophage protein	RPT			4,3	2,4
PA0618		probable bacteriophage protein	RPT			4,3	2,5
PA0619		probable bacteriophage protein	RPT			4,1	2,1
PA0620		probable bacteriophage protein	RPT			3,3	2,1
PA0621		conserved hypothetical protein	RPT			2,3	
PA0622		probable bacteriophage protein	RPT			4,8	2,4
PA0623		probable bacteriophage protein	RPT			4,1	2,0
PA0624		hypothetical protein	RPT			4,8	
PA0625		hypothetical protein	RPT			3,0	2,0
PA0626		hypothetical protein	RPT			2,7	
PA0627		conserved hypothetical protein	RPT			3,9	2,1
PA0628		conserved hypothetical protein	RPT			3,0	2,2
PA0629		conserved hypothetical protein	RPT			2,3	
PA0630		hypothetical protein	RPT			3,3	
PA0631		hypothetical protein	RPT			3,2	2,1
PA0633		hypothetical protein	RPT			3,1	
PA0634		hypothetical protein	RPT			4,4	2,1
PA0635		hypothetical protein	RPT			3,6	2,1
PA0636		hypothetical protein	RPT			4,9	3,2
PA0637		conserved hypothetical protein	RPT			4,4	2,3
PA0638		probable bacteriophage protein	RPT			4,5	2,9
PA0639		conserved hypothetical protein	RPT		-2,1	3,9	
PA0640		probable bacteriophage protein	RPT			2,2	
PA0641		probable bacteriophage protein	RPT			2,0	
PA0644		hypothetical protein	RPT			2,2	
PA0645		hypothetical protein	RPT			2,4	
PA0656		probable HIT family protein	PE	2,3			-4,7
PA0672	*hemO*	heme oxygenase	BC			2,7	4,0
PA0704		probable amidase	PE				-3,0
PA0705	*migA*	alpha-1,6-rhamnosyltransferase MigA	PE ; CLC			-2,2	
PA0707	*toxR*	transcriptional regulator ToxR	TR			2,7	3,0
PA0713		hypothetical protein	HUU		-6,2	3,4	
PA0732		hypothetical protein	HUU	2,0			-2,8
PA0734		hypothetical protein	HUU			-2,2	

PA0738		conserved hypothetical protein	MP			-2,7	-2,9
PA0741		conserved hypothetical protein	HUU				-2,1
PA0742		hypothetical protein	HUU	2,2			-2,8
PA0743		probable 3-hydroxyisobutyrate dehyd.*	CC	2,3			-2,6
PA0744		probable enoyl-CoA hydratase/isomerase	PE				-2,1
PA0745		probable enoyl-CoA hydratase/isomerase	PE	2,0			-2,4
PA0746		probable acyl-CoA dehydrogenase	PE	2,1			
PA0751		conserved hypothetical protein	MP			-2,0	-3,0
PA0752		conserved hypothetical protein	MP			-2,5	-5,0
PA0753		hypothetical protein	MP			-2,2	-3,8
PA0754		hypothetical protein	HUU			-4,7	-9,6
PA0755	*opdH*	cis-aconitate porin OpdH	MP; TSM			-2,0	-3,9
PA0760		conserved hypothetical protein	HUU				2,0
PA0764	*mucB*	negative regulator for alginate biosynthesis	TR ; CLC				-2,0
PA0767	*lepA*	GTP-binding protein LepA	TPD		2,1	-2,1	
PA0769		hypothetical protein	HUU				-2,4
PA0771	*era*	GTP-binding protein Era	CD ; TPD			-2,3	
PA0776		hypothetical protein	HUU				-2,6
PA0784		probable transcriptional regulator	TR				-3,0
PA0788		hypothetical protein	HUU	3,2			-4,5
PA0789		probable amino acid permease	MP; TSM				2,3
PA0791		probable transcriptional regulator	TR				-2,5
PA0793		hypothetical protein	HUU			2,1	3,1
PA0795	*prpC*	citrate synthase 2	CIM ; CC		2,0		2,8
PA0796	*prpB*	carboxyphosphonoenolpyruvate phosphonomutase	FPM ;CC			2,6	3,6
PA0797		probable transcriptional regulator	TR			2,3	4,1
PA0801		hypothetical protein	MP				2,4
PA0802		hypothetical protein	MP				3,5
PA0807	*ampD*	AmpDh3	ARS			2,0	
PA0829		probable hydrolase	PE			-2,0	-2,0
PA0830		hypothetical protein	HUU			-2,0	-2,7
PA0832		conserved hypothetical protein	HUU			-2,4	-3,2
PA0838		probable glutathione peroxidase	PE				-2,4
PA0839		probable transcriptional regulator	TR			-2,7	
PA0841		hypothetical protein	HUU			-2,7	
PA0847		hypothetical protein	MA				-2,2
PA0850		hypothetical protein	HUU			-2,5	-2,7
PA0853		probable oxidoreductase	PE				-3,3
PA0854	*fumC*	fumarate hydratase fumC2	EM ; CC				-2,1
PA0856		hypothetical protein	HUU		2,2	-3,3	
PA0861		hypothetical protein	MA				-2,4
PA0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase	ABM		2,6	6,2	11,8
PA0866	*aroP2*	aromatic amino acid transport protein AroP2	TSM		9,0		7,3
PA0867	*mliC*	m.-b.* lysozyme inhibitor of c-type lysozyme	Ap		3,0		2,7
PA0868		conserved hypothetical protein	HUU	-2,2			2,0
PA0869	*pbpG*	D-alanyl-D-alanine-endopeptidase	CLC			-2,1	-3,2
PA0870	*phhC*	aromatic amino acid aminotransferase	ABM			3,8	5,1
PA0871	*phhB*	pterin-4-alpha-carbinolamine dehydratase	ABM			2,8	4,0
PA0872	*phhA*	phenylalanine-4-hydroxylase	ABM			6,7	8,6
PA0876		probable transcriptional regulator	TR			-2,1	
PA0887	*acsA*	acetyl-coenzyme A synthetase	CC ; CIM		-5,4	3,5	
PA0888	*aotJ*	arginine/ornithine binding protein AotJ	TSM			-2,2	-2,2
PA0893	*argR*	transcriptional regulator ArgR	ABM; TR				-2,3
PA0895	*aruC*	N-succinylglutamate 5-semialdehyde dehyd.*	ABM				-3,0
PA0896	*aruF*	arginine/ornithine succinyltransferase AI sb.*	ABM				-2,6
PA0897	*aruG*	arginine/ornithine succinyltransferase AII sb.*	ABM	2,1	-2,1		-3,4
PA0898	*aruD*	succinylglutamate 5-semialdehyde dehyd.*	ABM	2,0	-2,0		-3,4
PA0899	*aruB*	succinylarginine dihydrolase	ABM	2,1	-2,2		-3,9
PA0900		hypothetical protein	HUU			-2,0	-2,7
PA0901	*aruE*	succinylglutamate desuccinylase	ABM		-2,1		-2,6
PA0904	*lysC*	aspartate kinase alpha and beta chain	ABM			-2,2	
PA0908		hypothetical protein	HUU			2,3	
PA0910		hypothetical protein	HUU			2,3	
PA0911		hypothetical protein	HUU			2,3	
PA0918		cytochrome b561	EM	2,3			-3,6
PA0919		hypothetical protein	HUU				-2,1
PA0925		hypothetical protein	HUU			-2,1	
PA0927	*ldhA*	D-lactate dehydrogenase (fermentative)	EM ; CC				-2,2
PA0929		two-component response regulator	TSM				2,0
PA0930		two-component sensor	TSM				2,2
PA0938		hypothetical protein	HUU				2,8
PA0942		probable transcriptional regulator	TR			2,0	
PA0952		hypothetical protein	HUU			-3,4	
PA0958	*oprD*	Basic amino acid, basic peptide and imipenem o.m.* porin OprD precursor	TSM				-2,5
PA0960		hypothetical protein	HUU				-2,1
PA0964	*pmpR*	pqsR-mediated PQS regulator, PmpR	HUU				2,0

PA0976		conserved hypothetical protein	HUU			2,2
PA0979		conserved hypothetical protein	RPT		2,2	
PA0989		hypothetical protein	HUU		-2,2	
PA0997	*pqsB*	Homolog. to beta-keto-acyl-acyl-carrier p.s.*	BC	2,3		
PA0998	*pqsC*	Homolog. to beta-keto-acyl-acyl-carrier p.s.*	BC	2,1		
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	BC	2,0		
PA1000	*pqsE*	Quinolone signal response protein	BC			2,4
PA1001	*phnA*	anthranilate synthase component I	Ap			2,6
PA1002	*phnB*	anthranilate synthase component II	ABM ; Ap			2,1
PA1009		hypothetical protein	HUU	2,1	-2,6	
PA1027		probable aldehyde dehydrogenase	PE		-2,4	-2,8
PA1033		probable glutathione S-transferase	PE		-2,4	
PA1039		conserved hypothetical protein	HUU			-2,3
PA1041		probable outer membrane protein precursor	MP; TSM			-3,4
PA1050		conserved hypothetical protein	HUU		2,6	-2,3
PA1057		conserved hypothetical protein	MP			-2,0
PA1058		conserved hypothetical protein	MP			-2,6
PA1061		conserved hypothetical protein	PE		-2,1	
PA1064		hypothetical protein	HUU		-2,1	
PA1071	*braF*	branched-chain amino acid transport protein	TSM			-2,1
PA1074	*braC*	branched-chain amino acid transport protein	TSM		-2,9	-3,7
PA1077	*flgB*	flagellar basal-body rod protein FlgB	CLC; MA		-2,2	-2,4
PA1078	*flgC*	flagellar basal-body rod protein FlgC	CLC; MA			-3,2
PA1079	*flgD*	flagellar b-b.* rod modification protein FlgD	CLC; MA			-2,7
PA1080	*flgE*	flagellar hook protein FlgE	CLC; MA		-2,4	-3,5
PA1081	*flgF*	flagellar basal-body rod protein FlgF	CLC; MA			-2,1
PA1082	*flgG*	flagellar basal-body rod protein FlgG	CLC; MA		-2,2	-2,9
PA1083	*flgH*	flagellar L-ring protein precursor FlgH	CLC; MA			-2,0
PA1086	*flgK*	flagellar hook-associated protein 1 FlgK	CLC; MA			-2,8
PA1087	*flgL*	flagellar hook-associated protein type 3 FlgL	CLC; MA			-2,8
PA1089		conserved hypothetical protein	HUU			-2,1
PA1090		hypothetical protein	HUU			-2,1
PA1092	*fliC*	flagellin type B	MA		-2,3	-2,8
PA1093		hypothetical protein	HUU			-2,5
PA1096		hypothetical protein	HUU	2,2		
PA1099	*fleR*	two-component response regulator	MA ; TR			-2,3
PA1116		hypothetical protein	HUU			2,2
PA1119		probable outer membrane protein precursor	MP			-2,1
PA1123		hypothetical protein	HUU	-5,9	8,0	
PA1130	*rhlC*	rhamnosyltransferase 2	CLC; Ap		3,0	3,9
PA1131		probable major facilitator superfamily tr.*	MP; ARS		4,5	4,0
PA1134		hypothetical protein	HUU		5,7	3,4
PA1135		conserved hypothetical protein	HUU	2,5		-4,0
PA1157		probable two-component response regulator	TR ; TCR			2,1
PA1166		hypothetical protein	HUU			-2,3
PA1167		hypothetical protein	HUU		-2,1	-5,2
PA1172	*napC*	cytochrome c-type protein NapC	EM	2,9		-5,7
PA1173	*napB*	cytochrome c-type protein NapB precursor	EM	3,4		-6,8
PA1174	*napA*	periplasmic nitrate reductase protein NapA	EM	3,0	-2,1	-6,8
PA1175	*napD*	NapD protein of periplasmic nitrate reductase	EM	3,1	-2,0	-6,9
PA1176	*napF*	ferredoxin protein NapF	EM	2,3	-2,2	-6,8
PA1177	*napE*	periplasmic nitrate reductase protein NapE	EM	-2,0		-2,6
PA1190		conserved hypothetical protein	MP	2,9	2,5	
PA1196		probable transcriptional regulator	TR		2,0	
PA1202		probable hydrolase	PE		-3,7	-5,2
PA1208		conserved hypothetical protein	HUU			-2,2
PA1216		hypothetical protein	HUU	2,1	6,1	-6,6	-2,3
PA1217		probable 2-isopropylmalate synthase	ABM		3,0		
PA1218		hypothetical protein	HUU		2,7		
PA1219		hypothetical protein	HUU		2,0		
PA1220		hypothetical protein	HUU		2,1		
PA1221		hypothetical protein	HUU		2,2		
PA1245		hypothetical protein	MP			4,4	3,5
PA1246	*aprD*	alkaline protease secretion protein AprD	SF ; PSE			2,7	
PA1247	*aprE*	alkaline protease secretion protein AprE	SF ; PSE			3,4	2,0
PA1249	*aprA*	alkaline metalloproteinase precursor	SF			2,5	
PA1250	*aprI*	alkaline proteinase inhibitor AprI	SF			2,1	2,3
PA1274		conserved hypothetical protein	HUU				2,2
PA1276	*cobC*	cobalamin biosynthetic protein CobC	BC	-2,1			2,3
PA1288		probable outer membrane protein precursor	MP; TSM			-4,2	-3,3
PA1289		hypothetical protein	HUU	3,3		-2,8	-7,9
PA1297		probable metal transporter	MP; TSM				2,1
PA1300		probable sigma-70 factor, ECF subfamily	TR		-2,2	11,8	8,4
PA1301		probable transmembrane sensor	MP ; TR		-2,3	7,9	4,6
PA1320	*cyoD*	cytochrome o ubiquinol oxidase subunit IV	EM			2,9	
PA1325		conserved hypothetical protein	HUU		2,6		3,0
PA1337	*ansB*	glutaminase-asparaginase	ABM	2,7		-2,9	-5,8
PA1338	*ggt*	gamma-glutamyltranspeptidase precursor	ABM ; Ap	2,6			-3,2

PA1339		amino acid ABC transporter ATP b.p.*	TSM			-3,3
PA1340		amino acid ABC transporter m.p.*	TSM	2,5		-3,8
PA1341		amino acid ABC transporter m.p.*	MP; TSM	2,3		-3,4
PA1342		probable b. p.* component of ABC transporter	TSM	2,3	-6,2	-8,0
PA1344		probable short-chain dehydrogenase	PE	2,0	-2,2	-5,1
PA1348		hypothetical protein	HUU	2,5		
PA1353		hypothetical protein	HUU	2,1		
PA1358		hypothetical protein	HUU	2,2		-2,9
PA1376	*aceK*	isocitrate dehydrogenase kinase/phosphatase	CIM		-2,1	-2,0
PA1382		probable type II secretion system protein	PSE		2,1	
PA1394		hypothetical protein	HUU	-2,2	-3,2	
PA1414		hypothetical protein	HUU	-3,1	5,0	
PA1415		hypothetical protein	HUU	2,2		-2,2
PA1418		probable sodium:solute symport protein	TSM			-2,2
PA1420		hypothetical protein	HUU			-2,3
PA1421	*gbuA*	guanidinobutyrase	ABM			-2,3
PA1429		probable cation-transporting P-type ATPase	MP; TSM	-3,2	5,7	2,0
PA1441		hypothetical protein	HUU			-2,1
PA1462		probable plasmid partitioning protein	CD			-2,0
PA1473		hypothetical protein	HUU	2,2		-2,3
PA1476	*ccmB*	heme exporter protein CcmB	MP; TSM			2,1
PA1483	*cycH*	cytochrome c-type biogenesis protein	EM		2,2	
PA1504		probable transcriptional regulator	TR	2,6	-2,7	
PA1525	*alkB2*	alkane-1-monooxygenase 2	CC		-2,1	-4,0
PA1535		probable acyl-CoA dehydrogenase	PE		-2,2	-3,6
PA1542		hypothetical protein	HUU			-2,1
PA1543	*apt*	adenine phosphoribosyltransferase	NM		-2,1	
PA1546	*hemN*	O2-independent coproporphyrinogen III ox.*	BC	-2,8	6,5	2,5
PA1548		conserved hypothetical protein	HUU	-2,3	2,4	
PA1550		hypothetical protein	HUU	-3,5	3,5	
PA1551		probable ferredoxin	EM	-2,6	2,7	
PA1555		probable cytochrome c	EM	-8,3	16,3	2,3
PA1556		probable cytochrome c oxidase subunit	EM	-6,7	13,9	2,3
PA1557		probable cytochr. oxidase sub.* (cbb3-type)	EM	-6,4	12,8	2,3
PA1558		hypothetical protein	HUU		2,1	
PA1559		hypothetical protein	HUU		-3,6	-3,4
PA1560		hypothetical protein	HUU			-2,1
PA1561	*aer*	aerotaxis receptor Aer	Ap ;C	-2,4	2,8	
PA1562	*acnA*	aconitate hydratase 1	EM			-2,0
PA1575		hypothetical protein	HUU			-2,4
PA1579		hypothetical protein	HUU		-2,3	
PA1580	*gltA*	citrate synthase	EM	2,7	-2,9	
PA1588	*sucC*	succinyl-CoA synthetase beta chain	EM	2,1	-2,0	
PA1590	*braB*	branched chain amino acid transporter	TSM		2,4	2,6
PA1591		hypothetical protein	MP			2,7
PA1592		hypothetical protein	HUU	2,1		-2,8
PA1593		hypothetical protein	HUU		-2,1	
PA1597		hypothetical protein	HUU		-2,2	
PA1610	*fabA*	beta-hydroxydecanoyl-ACP dehydrase	FPM			2,3
PA1617		probable AMP-binding enzyme	PE		-7,3	-10,0
PA1618		conserved hypothetical protein	HUU		-7,9	-5,9
PA1628		probable 3-hydroxyacyl-CoA dehydrogenase	PE			-2,1
PA1641		hypothetical protein	HUU			-2,5
PA1649		probable short-chain dehydrogenase	PE	-2,0	-2,7	
PA1650		probable transporter	MP; TSM			-4,3
PA1651		probable transporter	MP; TSM		-2,4	
PA1656		hypothetical protein	HUU	-2,3	3,7	
PA1672		hypothetical protein	HUU		2,3	
PA1673		hypothetical protein	HUU	-3,8	13,2	3,8
PA1674	*folE2*	GTP cyclohydrolase I precursor	BC	2,2	-2,1	
PA1711	*exsE*	ExsE	TR ; PSE		2,6	2,2
PA1712	*exsB*	exoenzyme S synthesis protein B	TPD		2,1	
PA1713	*exsA*	transcriptional regulator ExsA	PSE; TR		2,0	
PA1728		hypothetical protein	HUU	-2,1	2,7	
PA1733		conserved hypothetical protein	HUU	2,0		-2,6
PA1741		hypothetical protein	HUU			2,2
PA1745		hypothetical protein	HUU	3,1	-3,2	-9,4
PA1746		hypothetical protein	HUU	-8,8	27,3	2,5
PA1748		probable enoyl-CoA hydratase/isomerase	PE		-4,3	
PA1753		conserved hypothetical protein	HUU			-2,3
PA1754	*cysB*	transcriptional regulator CysB	ABM; TR			-2,4
PA1759		probable transcriptional regulator	TR		-2,4	-3,1
PA1760		probable transcriptional regulator	TR		-2,6	-3,5
PA1761		hypothetical protein	HUU		-8,0	-8,1
PA1762		hypothetical protein	HUU		-3,9	-5,7
PA1763		hypothetical protein	HUU	2,2	-6,7	-8,8
PA1764		hypothetical protein	HUU			-2,7
PA1765		hypothetical protein	HUU			-3,3

PA1773	*cmaX*	CmaX protein	MP				-2,2
PA1774	*crfX*	CrfX protein	HUU			2,4	2,0
PA1776	*sigX*	ECF sigma factor SigX	TR			2,2	2,2
PA1784		hypothetical protein	HUU	2,3			-3,1
PA1788		hypothetical protein	HUU				2,1
PA1800	*tig*	trigger factor	CD; CHP				2,6
PA1806	*fabI*	NADH-dependent enoyl-ACP reductase	FPM				-2,0
PA1807		probable ATP-binding component of ABC tr.*	TSM				-2,1
PA1811		probable solute-binding protein	TSM			-2,1	
PA1812	*mltD*	m.-b.* lytic murein transglycosylase D prc.*	ABM		2,6		
PA1818	*ldcA*	lysine-specific pyridoxal 5'-phosphate-dependent carboxylase, LdcA	ABM				-2,5
PA1828		probable short-chain dehydrogenase	PE			-5,8	-3,1
PA1829		hypothetical protein	PE			-3,5	-2,0
PA1830		hypothetical protein	HUU			-3,5	
PA1831		hypothetical protein	HUU			-2,1	
PA1837		hypothetical protein	HUU				2,5
PA1838	*cysI*	sulfite reductase	CIM				2,5
PA1842		hypothetical protein	HUU	-4,2		-2,5	2,2
PA1843	*metH*	methionine synthase	ABM	-4,8		-3,8	
PA1852		hypothetical protein	HUU			5,3	4,8
PA1853		probable transcriptional regulator	TR				2,4
PA1860		hypothetical protein	PE	2,2			-3,2
PA1869		probable acyl carrier protein	FPM	-2,3		7,4	19,0
PA1871	*lasA*	LasA protease precursor	SF; TPD			2,1	2,2
PA1874		hypothetical protein	ARS	4,1			-4,2
PA1875		probable outer membrane protein precursor	PSE	2,7			-2,0
PA1880		probable oxidoreductase	PE				-3,3
PA1881		probable oxidoreductase	PE	2,0			-2,9
PA1887		hypothetical protein	HUU	4,7			-8,0
PA1888		hypothetical protein	HUU	4,7			-7,6
PA1891		hypothetical protein	MP			2,9	3,6
PA1892		hypothetical protein	HUU			2,2	3,2
PA1893		hypothetical protein	PE			2,3	3,0
PA1894		hypothetical protein	HUU				2,5
PA1895		hypothetical protein	MP			2,7	2,9
PA1896		hypothetical protein	HUU			3,4	3,7
PA1897		hypothetical protein	MP				2,3
PA1898	*qscR*	quorum-sensing control repressor	TR		3,0		
PA1901	*phzC*	phenazine biosynthesis protein PhzC	SF		3,0	5,0	7,2
PA1902	*phzD*	phenazine biosynthesis protein PhzD	SF			6,4	5,9
PA1903	*phzE*	phenazine biosynthesis protein PhzE	SF			14,2	14,3
PA1904	*phzF2*	probable phenazine biosynthesis protein	SF			18,0	16,5
PA1905	*phzG*	probable pyridoxamine 5'-phosphate oxidase	SF			21,8	20,0
PA1914		conserved hypothetical protein	PE	2,3			-2,5
PA1927	*metE*	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	ABM	-168,3	-59,5	-2,1	
PA1930		probable Ctransducer	Ap ;C	3,0			-4,4
PA1941		hypothetical protein	HUU			-2,8	
PA1943		hypothetical protein	HUU				-2,2
PA1944		hypothetical protein	HUU				-3,2
PA1946	*rbsB*	b.p.* component precursor of ABC ribose tr.*	TSM				-2,5
PA1947	*rbsA*	ribose transport protein RbsA	TSM				-2,1
PA1951		hypothetical protein	HUU	2,3			-4,8
PA1965		hypothetical protein	HUU	-2,1			2,6
PA1967		hypothetical protein	HUU			-2,1	-2,8
PA1971	*braZ*	branched chain amino acid transporter BraZ	TSM			-2,3	
PA1975		hypothetical protein	HUU			-3,1	-2,1
PA1977		hypothetical protein	MP				-2,1
PA1978	*erbR*	response regulator ErbR	TR			-7,3	-9,2
PA1979	*eraS*	sensor kinase, EraS	TCR			-3,0	-2,5
PA1980	*eraR*	response regulator EraR	TR ; TCR			-2,0	-2,1
PA1983	*exaB*	cytochrome c550	EM ; CC			-2,2	
PA1984	*exaC*	NAD+ dependent aldehyde dehyd. ExaC	PE	3,7		-2,4	-7,6
PA1985	*pqqA*	pyrroloquinoline quinone bio. p.* A	BC			-2,1	-2,6
PA1986	*pqqB*	pyrroloquinoline quinone bio. p.*B	BC			-2,4	-3,2
PA1987	*pqqC*	pyrroloquinoline quinone bio. p.* C	BC				-2,1
PA1988	*pqqD*	pyrroloquinoline quinone bio. p.*D	BC			-2,1	-2,2
PA1989	*pqqE*	pyrroloquinoline quinone bio. p.* E	BC				-2,0
PA1999	*dhcA*	DhcA, dehydrocarnitine CoA transf., sub.* A	ABM; CC			32,6	29,3
PA2000	*dhcB*	DhcB, dehydrocarnitine CoA transf., sub.* B	ABM ;CC			11,4	18,4
PA2001	*atoB*	acetyl-CoA acetyltransferase	CIM			6,9	11,3
PA2002		conserved hypothetical protein	MP		2,4		3,0
PA2003	*bdhA*	3-hydroxybutyrate dehydrogenase	CC			4,2	2,8
PA2004		conserved hypothetical protein	MP			4,0	3,9
PA2006		probable major facilitator superfamily tr.*	MP; TSM		2,9	2,2	7,0
PA2007	*maiA*	maleylacetoacetate isomerase	CC			22,0	14,5
PA2008	*fahA*	fumarylacetoacetase	CC			9,4	8,2

PA2009	*hmgA*	homogentisate 1,2-dioxygenase	CC			10,2	17,3
PA2010		probable transcriptional regulator	TR		-2,6	7,9	3,8
PA2011	*liuE*	3-hydroxy-3-methylglutaryl-CoA lyase	CC			5,4	7,3
PA2012	*liuD*	methylcrotonyl-CoA carboxylase, alpha-subunit (biotin-containing)	CC CC			9,7	7,6
PA2013	*liuC*	putative 3-methylglutaconyl-CoA hydratase				4,3	5,7
PA2014	*liuB*	methylcrotonyl-CoA carboxylase, beta-subunit	CC			10,5	10,6
PA2015	*liuA*	putative isovaleryl-CoA dehydrogenase	CC			11,7	12,5
PA2016	*liuR*	regulator of liu genes	TR			6,4	12,1
PA2024		probable ring-cleaving dioxygenase	PE	2,5	-2,1		-3,6
PA2027		hypothetical protein	HUU		2,3	9,7	25,3
PA2030		hypothetical protein	HUU		2,0		
PA2031		hypothetical protein	HUU		3,0		
PA2033		hypothetical protein	HUU			2,9	2,2
PA2034		hypothetical protein	HUU		-2,3	7,8	4,1
PA2038		hypothetical protein	HUU				2,7
PA2047		probable transcriptional regulator	TR				-2,0
PA2062		probable pyridoxal-phosphate dep.* enzyme	PE			2,4	
PA2067		probable hydrolase	PE			2,5	
PA2068		probable major facilitator superfamily tr.*	MP; TSM			2,2	3,4
PA2069		probable carbamoyl transferase	PE			3,3	4,0
PA2071	*fusA2*	elongation factor G	TPD	2,7			-4,3
PA2072		conserved hypothetical protein	MP				-4,5
PA2080	*kynU*	kynureninase KynU	HUU		-2,3	-2,7	
PA2081	*kynB*	kynurenine formamidase, KynB	ABM		2,1	-2,3	
PA2109		hypothetical protein	HUU		2,5	-4,6	-10,3
PA2110		hypothetical protein	HUU			-10,5	-19,4
PA2111		hypothetical protein	HUU			-12,1	-27,9
PA2112		conserved hypothetical protein	HUU			-16,5	-32,8
PA2113	*opdO*	pyroglutamate porin OpdO	TSM; MP	2,4		-6,6	-17,9
PA2114		probable major facilitator superfamily tr.*	MP; TSM	2,2		-10,1	-31,2
PA2116		conserved hypothetical protein	HUU	2,3		-6,2	-16,2
PA2119		alcohol dehydrogenase (Zn-dependent)	PE			3,7	
PA2120		hypothetical protein	HUU		-2,5		-2,2
PA2121		probable transcriptional regulator	TR				-2,5
PA2128	*cupA*	fimbrial subunit CupA1	MA	3,2			-2,6
PA2174		hypothetical protein	HUU	2,8			-3,0
PA2177		probable sensor/response regulator hybrid	TCR				-2,1
PA2182		hypothetical protein	HUU	2,6			-2,8
PA2193	*hcnA*	hydrogen cyanide synthase HcnA	CIM			14,9	21,4
PA2194	*hcnB*	hydrogen cyanide synthase HcnB	CIM			14,0	10,3
PA2195	*hcnC*	hydrogen cyanide synthase HcnC	CIM			9,9	12,0
PA2204		probable binding protein com. of ABC tr.*	TSM			2,5	
PA2205		hypothetical protein	HUU			-3,0	
PA2231	*pslA*	PslA	CLC				-2,4
PA2232	*pslB*	PslB	CLC				-3,2
PA2233	*pslC*	PslC	PE ; CLC	2,1			-2,9
PA2234	*pslD*	PslD	CLC	2,2			-2,8
PA2235	*pslE*	PslE	CLC				-2,2
PA2236	*pslF*	PslF	CLC				-2,3
PA2237	*pslG*	PslG	PE ; CLC				-2,2
PA2239	*pslI*	PslI	PE ; CLC				-2,1
PA2242	*pslL*	hypothetical protein	HUU				-2,1
PA2247	*bkdA*	2-oxoisovalerate dehyd. (alpha subunit)	ABM			3,3	5,8
PA2248	*bkdA*	2-oxoisovalerate dehyd. (beta subunit)	ABM			5,4	6,6
PA2249	*bkdB*	branched-chain alpha-keto acid dehyd.	ABM			6,3	8,9
PA2250	*lpdV*	lipoamide dehydrogenase-Val	ABM; EM			7,8	8,9
PA2273		probable transcriptional regulator	TR			2,4	2,1
PA2274		hypothetical protein	HUU			2,5	7,0
PA2290	*gcd*	glucose dehydrogenase	CC ; EM		2,3		-2,0
PA2302		probable non-ribosomal peptide synthetase	PE			2,5	2,2
PA2305		probable non-ribosomal peptide synthetase	PE				2,2
PA2306		conserved hypothetical protein	MP			-2,3	
PA2326		hypothetical protein	PE			2,1	
PA2327		probable permease of ABC transporter	MP; TSM			2,2	
PA2329		probable ATP-binding component of ABC tr.*	TSM			2,1	
PA2357	*msuE*	NADH-dependent FMN reductase MsuE	CC ; CIM			-4,0	-4,1
PA2364		hypothetical protein	HUU			-2,3	-2,8
PA2365		conserved hypothetical protein	HUU				-2,8
PA2366		uricase PuuD	HUU				-2,9
PA2367		hypothetical protein	HUU				-2,6
PA2371		probable ClpA/B-type protease	TPD				-2,5
PA2372		hypothetical protein	HUU				-2,5
PA2373		conserved hypothetical protein	HUU				-2,5
PA2379		probable oxidoreductase	PE				2,2
PA2380		hypothetical protein	HUU		-2,6	3,9	
PA2381		hypothetical protein	HUU			3,4	2,1
PA2382	*lldA*	L-lactate dehydrogenase	EM			2,4	2,0

PA2383		probable transcriptional regulator	TR			5,9	4,9
PA2384		hypothetical protein	HUU			6,8	6,5
PA2385	*pvdQ*	3-oxo-C12-homoserine lactone acylase PvdQ	Ap			18,8	18,8
PA2386	*pvdA*	L-ornithine N5-oxygenase	Ap				2,7
PA2388	*fpvR*	FpvR	MP ; TR			4,6	4,3
PA2389		conserved hypothetical protein	HUU			4,2	3,1
PA2390		probable ATP-binding/permease fusion ABC	MP; TSM			3,7	2,8
PA2391	*opmQ*	transporter	MP; TSM			10,5	8,8
PA2392	*pvdP*	probable o. m.* protein precursor PvdP	Ap			5,3	6,0
PA2393		probable dipeptidase precursor	CIM			7,2	7,9
PA2394	*pvdN*	PvdN	Ap			2,9	3,1
PA2395	*pvdO*	PvdO	Ap			9,1	10,5
PA2396	*pvdF*	pyoverdine synthetase F	SF ; Ap			6,2	6,9
PA2397	*pvdE*	pyoverdine biosynthesis protein PvdE	MP ; Ap			2,0	3,7
PA2398	*fpvA*	ferripyoverdine receptor	TSM			18,7	12,9
PA2399	*pvdD*	pyoverdine synthetase D	SF ; Ap			15,5	8,7
PA2400	*pvdJ*	PvdJ	Ap			14,8	9,1
PA2402		probable non-ribosomal peptide synthetase	PE			13,5	10,9
PA2404		hypothetical protein	MP			2,4	2,9
PA2405		hypothetical protein	HUU			2,3	2,5
PA2406		hypothetical protein	HUU			2,4	2,4
PA2407		probable adhesion protein	MA			2,2	2,6
PA2408		probable ATP-binding com.* of ABC tr.*	TSM			3,4	3,0
PA2411		probable thioesterase	Ap ; PE		-2,0	21,1	15,1
PA2412		conserved hypothetical protein	HUU			17,3	17,9
PA2413	*pvdH*	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	Ap		-2,1	14,4	8,3
PA2424	*pvdL*	PvdL	Ap		-2,1	21,6	11,4
PA2425	*pvdG*	PvdG	Ap			8,9	7,6
PA2426	*pvdS*	sigma factor PvdS	TR		2,2	2,6	7,6
PA2427		hypothetical protein	HUU			23,9	14,2
PA2433		hypothetical protein	HUU	2,2			-2,5
PA2445	*gcvP2*	glycine cleavage system protein P2	CIM				2,3
PA2446	*gcvH*	glycine cleavage system protein H2	ABM				2,5
PA2450		hypothetical protein	HUU				2,2
PA2451		hypothetical protein	HUU			2,8	
PA2453		hypothetical protein	HUU			-2,3	
PA2464		hypothetical protein	HUU	-2,1			2,2
PA2467	*foxR*	Anti-sigma factor FoxR	MP ; TR			3,7	2,9
PA2468	*foxI*	ECF sigma factor FoxI	TR				2,5
PA2481		hypothetical protein	HUU				-2,3
PA2486		hypothetical protein	HUU	2,3			-2,5
PA2501		hypothetical protein	MP		-2,5	3,0	
PA2504		hypothetical protein	HUU	2,2			-2,2
PA2511		probable transcriptional regulator	TR		4,8		3,8
PA2512	*antA*	anthranilate dioxygenase large subunit	CC		3,4		3,7
PA2513	*antB*	anthranilate dioxygenase small subunit	CC		5,3		5,9
PA2514	*antC*	anthranilate dioxygenase reductase	CC		4,2		5,1
PA2532	*tpx*	thiol peroxidase	Ap		2,3	-2,3	
PA2539		conserved hypothetical protein	MP				2,3
PA2550		probable acyl-CoA dehydrogenase	PE	-2,0		-2,1	
PA2552		probable acyl-CoA dehydrogenase	PE				2,1
PA2553		probable acyl-CoA thiolase	PE			2,5	2,7
PA2554		probable short-chain dehydrogenase	PE			2,7	3,0
PA2555		probable AMP-binding enzyme	PE			2,5	3,5
PA2556		probable transcriptional regulator	TR			2,1	2,9
PA2557		probable AMP-binding enzyme	FPM			4,5	7,4
PA2558		probable transport protein	MP; TSM				2,5
PA2562		hypothetical protein	HUU	2,5			-2,6
PA2564		hypothetical protein	HUU			6,4	3,3
PA2565		hypothetical protein	HUU			4,6	2,8
PA2566		conserved hypothetical protein	HUU	2,1		7,3	2,6
PA2568		hypothetical protein	MP				2,1
PA2572		probable two-component response regulator	TR ; TCR	2,1			-2,6
PA2573		probable Ctransducer	Ap ;C				-2,1
PA2577		probable transcriptional regulator	TR	2,1		-2,2	-3,8
PA2578		probable acetyltransferase	PE				-3,2
PA2579	*kynA*	L-Tryptophan:oxygen 2,3-oxidoreductase KynA	PE		2,5		
PA2591		probable transcriptional regulator prob.*periplasmic spermidine/putrescine-b. p.*	TR			3,3	2,1
PA2592		hypothetical protein	TSM			3,5	3,6
PA2593		conserved hypothetical protein	HUU			2,3	3,1
PA2605		conserved hypothetical protein	HUU				-2,1
PA2606		conserved hypothetical protein	HUU				-2,0
PA2607		conserved hypothetical protein	HUU				-2,4
PA2608		hypothetical protein	HUU				-2,5
PA2609		hypothetical protein	HUU				-2,6
PA2618		initiation factor	TPD	2,2			-2,7

PA2619	*infA*	adenylosuccinate lyase	TPD	2,1		2,2
PA2629	*purB*	hypothetical protein	ABM; NM	2,0		2,2
PA2633		isocitrate lyase AceA	RPT			-2,2
PA2634	*aceA*	NADH dehydrogenase I chain C,D	PE		-3,0	
PA2639	*nuoD*	NADH dehydrogenase I chain E	EM	2,2	-2,6	
PA2640	*nuoE*	NADH Dehydrogenase I chain I	EM	2,0		
PA2644	*nuoI*	NADH dehydrogenase I chain K	EM	2,3	-2,1	
PA2646	*nuoK*	hypothetical protein	EM	2,0		
PA2658		hypothetical protein	HUU			-2,3
PA2659		conserved hypothetical protein	HUU			-2,6
PA2662		conserved hypothetical protein	MP	-2,2	3,0	
PA2667		conserved hypothetical protein	HUU	2,2		
PA2682		probable transcriptional regulator	PE	4,2		3,7
PA2704		chloroperoxidase precursor	TR	2,3	-3,6	-2,2
PA2717	*cpo*	50S ribosomal protein L20	CIM			-2,2
PA2741	*rplT*	threonyl-tRNA synthetase	TPD			2,4
PA2744	*thrS*	hypothetical protein	ABM		-2,0	
PA2746		hypothetical protein	MP		2,9	2,5
PA2747		hypothetical protein	HUU		2,6	
PA2753		conserved hypothetical protein	HUU		2,4	
PA2754		hypothetical protein	HUU	-2,1	3,4	
PA2756		probable outer membrane protein precursor	HUU	-2,2	2,4	
PA2760		hypothetical protein	TSM	2,2		
PA2761		hypothetical protein	MP	-2,2	-2,1	
PA2762		conserved hypothetical protein	HUU	2,8		-3,0
PA2771		hypothetical protein	HUU			-3,3
PA2774		hypothetical protein	MP	-2,3		2,4
PA2775		conserved hypothetical protein	MP			2,2
PA2776		hypothetical protein	HUU			-2,0
PA2779		probable Ctransducer	HUU	2,4		-4,8
PA2788		conserved hypothetical protein	Ap ;C	-2,6	4,9	
PA2822		probable sensor/response regulator hybrid	HUU			2,1
PA2824		OspR	TCR			-2,5
PA2825	*ospR*	probable glutathione peroxidase	TR			-3,0
PA2826		probable enoyl-CoA hydratase/isomerase	Ap		-2,3	-3,1
PA2841		hypothetical protein	PE			-2,4
PA2842		conserved hypothetical protein	HUU			2,4
PA2844		hypothetical protein	HUU		2,2	
PA2845		conserved hypothetical protein	HUU	-4,7	4,8	
PA2847		probable transcriptional regulator	HUU		2,0	
PA2849		translation elongation factor P	TR		-2,3	-2,6
PA2851	*efp*	lactonizing lipase precursor	TPD			2,7
PA2862	*lipA*	lipase modulator protein	CC ; SF	4,4	-50,9	-20,0
PA2863	*lipH*	conserved hypothetical protein	PSE; SF		-3,1	-3,8
PA2864		probable Ctransducer	HUU			-2,1
PA2867		orotidine 5'-phosphate decarboxylase	Ap ;C		3,2	
PA2876	*pyrF*	citronellol catabolism	NM			2,2
PA2886	*atuA*	putative dehydrogenase	HUU	2,2	-2,0	-4,5
PA2887	*atuB*	geranyl-CoA carboxylase, beta-subunit	PE	2,1		-4,2
PA2888	*atuC*	putative citronellyl-CoA dehydrogenase	PE	2,9		-5,0
PA2889	*atuD*	putative isohexenylglutaconyl-CoA hydratase	PE	3,7		-6,7
PA2890	*atuE*	geranyl-CoA carboxylase, alpha-subunit	PE	2,5		-4,2
PA2891	atuF	GCase, alpha-subunit (biotin-containing)	PE	2,8		-5,6
PA2892	atuG	putative very-long chain acyl-CoA synthetase	PE	4,1	-3,7	-13,8
PA2893	*atuH*	probable outer membrane protein precursor	FPM	2,1		-2,5
PA2900		hypothetical protein	MP; TSM			2,1
PA2901		probable oxidoreductase	HUU			3,0
PA2906		hypothetical protein	PE	-2,1		2,3
PA2929		CifR	MP	-2,4		
PA2931	*cifR*	hypothetical protein	TR		2,1	
PA2937		probable aminopeptidase	HUU		2,1	
PA2939		hypothetical protein	SF	2,5		-4,4
PA2941		conserved hypothetical protein	HUU			2,3
PA2945		hypothetical protein	BC	-2,0		2,1
PA2950		electron transfer flavoprotein beta-subunit	HUU			2,4
PA2952	*etfB*	e.* transfer fp*-ubiquinone oxidoreductase	EM		-2,2	
PA2953		3-oxoacyl-[acyl-carrier-protein] reductase	EM		-2,1	
PA2967	*fabG*	malonyl-CoA- transacylase	FPM	-2,0	6,2	3,3
PA2968	*fabD*	50S ribosomal protein L32	FPM			2,7
PA2970	*rpmF*	conserved hypothetical protein	TPD	-2,0		2,8
PA2971		conserved hypothetical protein	HUU	-2,4		3,0
PA2982		probable tolQ-type transport protein	HUU	-2,1		2,3
PA2983		conserved hypothetical protein	TSM			2,2
PA2993		NADH:quinone oxidoreductase, subunit Nqr6	HUU			2,2
PA2994	*nqrF*	NADH:uniquinone oxidoreductase sub.* Nqr4	EM			2,1
PA2996	*nqrD*	aromatic amino acid transport protein AroP1	EM			2,1
PA3000	*aroP1*	probable glyceraldehyde-3-phosphate dehyd.*	TSM			2,3
PA3001		transcriptional regulator PsrA	PE			2,1

PA3006	*psrA*	hypothetical protein	TR	-2,3			2,6
PA3009		conserved hypothetical protein	HUU				2,3
PA3017		probable ATP-binding component of ABC tr.*	HUU			-2,3	
PA3019		hypothetical protein	TSM				2,3
PA3021		hypothetical protein	HUU			-3,4	-2,2
PA3022		probable porin	HUU			2,1	
PA3038		conserved hypothetical protein	TSM		-2,3		-2,5
PA3040		hypothetical protein	HUU	2,4			-4,7
PA3041		hypothetical protein	MP	2,0			-3,8
PA3042		ribosome modulation factor	HUU				-3,0
PA3049	*rmf*	hypothetical protein	TPD	3,4		3,5	
PA3079		hypothetical protein	MP			-6,4	-3,9
PA3080		conserved hypothetical protein	HUU			-9,7	-5,4
PA3081		hypothetical protein	HUU			-3,9	-2,7
PA3089		2,4-dienoyl-CoA reductase FadH1	HUU				-2,4
PA3092	*fadH1*	folylpolyglutamate synthetase	FPM			-2,7	-2,4
PA3111	*folC*	acetyl-CoA carboxylase beta subunit	BC				2,7
PA3112	*accD*	N-(5'phosphoribosyl)anthranilate isomerase	FPM				2,4
PA3113	*trpF*	probable aspartate-semialdehyde dehyd.*	ABM				2,3
PA3116		conserved hypothetical protein	ABM				2,5
PA3123		conserved hypothetical protein	HUU				-2,5
PA3129		hypothetical protein	HUU				3,0
PA3142		30S ribosomal protein S1	RPT			2,1	
PA3162	*rpsA*	prephenate dehydrogenase	TPD				2,7
PA3164		histidinol-phosphate aminotransferase	ABM				2,0
PA3165	*hisC2*	5-methylthioribose-1-phosphate isomerase	ABM				2,2
PA3169	*mtnA*	hypothetical protein	TPD				2,2
PA3177		hypothetical protein	HUU				-2,1
PA3178		2-keto-3-deoxy-6-phosphogluconate aldolase	HUU				2,8
PA3181		6-phosphogluconolactonase	CIM ; CC				-2,0
PA3182	*pgl*	glucose-6-phosphate 1-dehydrogenase	CIM	2,1			-2,7
PA3183	*zwf*	Glucose/carbohydrate outer membrane porin	EM ; CC	3,6			-4,7
PA3186	*oprB*	probable permease of ABC sugar transporter	TSM		-2,1		-2,7
PA3188		prob.* binding protein com.* of ABC sugar tr.*	TSM				-2,4
PA3190		glyceraldehyde 3-phosphate dehydrogenase	TSM			-2,0	-3,3
PA3195	*gapA*	conserved hypothetical protein	EM ; CC	5,1			-8,4
PA3199		hypothetical protein	HUU			-2,0	
PA3216		peptidyl-prolyl cis-trans isomerase A	MP	2,1			-3,3
PA3227	*ppiA*	probable sodium:solute symporter	TPD			-2,9	-3,4
PA3234		conserved hypothetical protein	MP; TSM		-3,4		-2,5
PA3235		probable glycine betaine-binding protein	MP		-4,4	2,9	-2,1
PA3236		precursor	Ap ; TSM	4,1			-6,5
PA3242		probable lauroyl acyltransferase	CLC				2,2
PA3245	*minE*	hypothetical protein	CD				2,1
PA3250		hypothetical protein	HUU	3,2		-2,1	-6,4
PA3251		probable permease of ABC transporter	HUU				-2,0
PA3252		probable ATP-binding component of ABC tr.*	MP; TSM				-2,4
PA3254		conserved hypothetical protein	TSM				-2,1
PA3263		hypothetical protein	HUU			-2,5	
PA3270		hypothetical protein	HUU				2,2
PA3276		probable short-chain dehydrogenase	HUU				2,2
PA3277		hypothetical protein	PE			-3,1	-3,3
PA3278		probable sigma-70 factor, ECF subfamily	MP	-2,6	-2,5		
PA3285		hypothetical protein	TR				2,3
PA3289		probable HIT family protein	HUU				-3,1
PA3295		long-chain-fatty-acid--CoA ligase	PE			-2,3	
PA3299	*fadD1*	long-chain-fatty-acid--CoA ligase	FPM			-3,2	
PA3300	*fadD2*	conserved hypothetical protein	FPM			-2,0	
PA3309		conserved hypothetical protein	HUU		-4,9	12,1	2,2
PA3311		conserved hypothetical protein	MP				-2,3
PA3323		conserved hypothetical protein	HUU				-2,5
PA3325		probable Clp-family ATP-dependent protease	HUU				-2,0
PA3326		probable non-ribosomal peptide synthetase	TPD			6,6	5,6
PA3327		probable FAD-dependent monooxygenase	Ap			5,0	7,0
PA3328		hypothetical protein	PE			5,0	5,4
PA3329		probable short chain dehydrogenase	HUU			4,3	6,9
PA3330		cytochrome P450	PE			9,8	8,3
PA3331		conserved hypothetical protein	Ap ; CC			8,1	8,1
PA3332		3-oxoacyl-[acyl-carrier-protein] synthase III	HUU			6,7	9,6
PA3333	*fabH2*	probable acyl carrier protein	FPM			8,7	8,2
PA3334		hypothetical protein	FPM			14,6	14,7
PA3335		probable major facilitator superfamily tr.*	HUU			12,3	12,3
PA3336		ADP-L-glycero-D-mannoheptose 6-epimerase	MP; TSM			8,9	7,4
PA3337	*rfaD*	hypothetical protein	CLC		-3,0	5,6	
PA3342		ATP-dependent DNA helicase RecQ	MP				-2,3
PA3344	*recQ*	hypothetical protein	DRR				2,1
PA3347		fucose-binding lectin PA-IIL	HUU				-2,0
PA3361	*lecB*	hypothetical protein	MA			33,1	61,2

PA3362		aliphatic amidase regulator	MP			-4,5	-11,0
PA3363	*amiR*	aliphatic amidase expression-regulating p.*	CC; TR	2,3		-5,8	-13,4
PA3364	*amiC*	probable chaperone	TR			-4,8	-9,8
PA3365		aliphatic amidase	CHP			-5,8	-10,6
PA3366	*amiE*	beta-ketoacyl reductase	CC	2,2		-11,6	-21,2
PA3387	*rhlG*	ferredoxin--NADP+ reductase	CLC			-2,4	
PA3397	*fpr*	heme acquisition protein HasAp	BC ; EM				4,3
PA3407	*hasA*	hypothetical protein	TSM			4,2	2,3
PA3412		probable pyruvate dehydrogenase E1 com.*	HUU		-2,0	2,8	
PA3416		leucine dehydrogenase	EM	2,4		2,4	
PA3418	*ldh*	probable transcriptional regulator	ABM	2,7			-3,9
PA3420		conserved hypothetical protein	TR			-3,5	-2,7
PA3421		hypothetical protein	HUU	2,2		-2,7	-6,0
PA3422		probable short-chain dehydrogenases	HUU			-2,2	-3,8
PA3427		probable epoxide hydrolase	PE			-4,2	-2,4
PA3429		probable aldolase	PE			-2,1	-2,3
PA3430		conserved hypothetical protein	PE			-2,4	-3,3
PA3435		conserved hypothetical protein	HUU		2,1	-2,0	
PA3440		hypothetical protein	HUU		2,0	-2,8	
PA3451		probable acyl-CoA thiolase	HUU	2,9			-2,3
PA3454		conserved hypothetical protein	PE			-3,1	-2,2
PA3465		hypothetical protein	MP	2,6			-3,8
PA3472		cyclohexadienyl dehydratase precursor	HUU				2,6
PA3475	*pheC*	autoinducer synthesis protein RhlI	Ap ; ABM				2,2
PA3476	*rhlI*	rhamnosyltransferase chain B	Ap				2,8
PA3478	*rhlB*	rhamnosyltransferase chain A	SF			7,8	3,5
PA3479	*rhlA*	hypothetical protein	SF			10,4	10,6
PA3496		hypothetical protein	HUU			2,6	2,9
PA3520		argininosuccinate synthase	HUU			5,1	2,9
PA3525	*argG*	conserved hypothetical protein	ABM		2,3		
PA3530		hypothetical protein	HUU			2,2	4,7
PA3536		probable acetyl-coa synthetase	HUU				2,2
PA3568		3-hydroxyisobutyrate dehydrogenase	PE	2,0			
PA3569	*mms*	methylmalonate-semialdehyde dehyd.*	CC		-2,0	3,2	
PA3570	*mms*	hypothetical protein *mmsA*	ABM; CC		-2,2	3,2	
PA3572		glycerol uptake facilitator protein	HUU			6,6	2,9
PA3581	*glpF*	glycerol kinase	TSM			-7,4	-5,9
PA3582	*glpK*	glycerol-3-phosphate regulon repressor	CIM			-8,3	-9,9
PA3583	*glpR*	glycerol-3-phosphate dehydrogenase	TR			-2,0	
PA3584	*glpD*	transcriptional regulator MetR	CIM ; EM			-2,1	
PA3587	*metR*	hypothetical protein	TR	-3,3	-3,1		
PA3611		hypothetical protein	HUU			-2,2	
PA3613		conserved hypothetical protein	HUU			2,5	
PA3618		probable esterase	HUU				2,4
PA3628		alcohol dehydrogenase class III	PE				-2,4
PA3629	*adhC*	probable amino acid permease	CIM			-2,1	-2,8
PA3641		ribonuclease HII	TSM				3,1
PA3642	*rnhB*	UDP-N-acetylglucosamine acyltransferase	DRR				2,1
PA3644	*lpxA*	1-deoxy-d-xylulose 5-phosphate	CLC				2,3
PA3650	*dxr*	reductoisomerase	BC				2,1
PA3651	*cdsA*	phosphatidate cytidylyltransferase uridylate kinase	FPM				2,1
PA3654	*pyrH*	elongation factor Ts	NM				2,5
PA3655	*tsf*	30S ribosomal protein S2	TPD		2,6		2,9
PA3656	*rpsB*	hypothetical protein	TPD		2,5		2,7
PA3661		hypothetical protein	HUU			-3,2	
PA3662		hypothetical protein	HUU			2,3	
PA3688		hypothetical protein	HUU	2,9			-3,0
PA3691		probable major facilitator superfamily tr.*	HUU	2,0			
PA3709		probable GMC-type oxidoreductase	MP; TSM	3,4	2,1	-2,5	-3,9
PA3710		hypothetical protein	CC	2,2			
PA3712		probable FMN oxidoreductase	HUU				-2,2
PA3723		probable FMN oxidoreductase	PE	3,3			
PA3723		probable FMN oxidoreductase	PE			-2,4	
PA3723		elastase LasB	PE				-8,6
PA3724	*lasB*	conserved hypothetical protein	SF ; TPD			6,9	14,5
PA3731		50S ribosomal protein L19	HUU	-2,2			
PA3742	*rplS*	tRNA (guanine-N1)-methyltransferase	TPD	-2,4	2,2		4,8
PA3743	*trmD*	16S rRNA processing protein	TRD	-2,1			4,4
PA3744	*rimM*	30S ribosomal protein S16	TRD	-2,0			3,4
PA3745	*rpsP*	signal recognition particle protein Ffh	DRR	-2,2			3,3
PA3746	*ffh*	probable major facilitator superfamily tr.*	PSE				2,1
PA3749		probable metallo-oxidoreductase	MP; TSM			-2,6	-2,3
PA3768		hypothetical protein	PE				2,1
PA3784		conserved hypothetical protein	HUU				-4,3
PA3785		hypothetical protein	HUU				-3,2
PA3786		hypothetical protein	HUU			2,0	
PA3791		probable oxidoreductase	MP		-2,7	2,4	

PA3795		conserved hypothetical protein	PE				-2,2
PA3808		ferredoxin [2Fe-2S]	HUU			2,2	
PA3809	*fdx2*	heat shock protein HscA	EM				2,5
PA3810	*hscA*	heat shock protein HscB	CHP				3,0
PA3811	*hscB*	probable iron-binding protein IscA	CHP				2,3
PA3812	*iscA*	probable iron-binding protein IscU	BC				2,2
PA3813	*iscU*	L-cysteine desulphurase	BC		2,4		
PA3814	*iscS*	conserved hypothetical protein	ABM; BC		2,3		
PA3815		O-acetylserine synthase	HUU		2,0		2,3
PA3816	*cysE*	probable methyltransferase	BC; ABM				2,3
PA3817		conserved hypothetical protein	PE				2,1
PA3819		secretion protein SecF	MP	2,3	-2,4	3,1	
PA3820	*secF*	conserved hypothetical protein	PSE				2,1
PA3822		SAM-:trna ribosyltransferase-isomerase	HUU				2,2
PA3824	*queA*	conserved hypothetical protein	TPD				2,1
PA3827		hypothetical protein	MP				2,2
PA3833		hypothetical protein	HUU	2,2			-3,1
PA3835		hypothetical protein	HUU			2,1	
PA3836		hypothetical protein	HUU			-2,5	
PA3848		phosphatidylcholine synthase	HUU				-2,9
PA3857	*pcs*	probable amino acid-binding protein	FPM				-2,6
PA3858		probable AMP-binding enzyme	TSM		-2,1		-2,2
PA3860		two-component response regulator NarL	PE			-4,8	-4,9
PA3879	*narL*	conserved hypothetical protein	EM; TCR			2,4	
PA3880		probable protease	HUU		-2,1	3,8	
PA3913		conserved hypothetical protein	PE			2,2	
PA3919		conserved hypothetical protein	HUU	3,4			-5,1
PA3922		hypothetical protein	HUU	2,1	2,3	-11,8	-10,9
PA3923		probable medium-chain acyl-CoA ligase	HUU		2,2	-10,2	-8,3
PA3924		probable acyl-CoA thiolase	FPM			-4,8	-5,8
PA3925		cyanide insensitive terminal oxidase	PE	-2,4		-5,5	
PA3930	*cioA*	conserved hypothetical protein	EM				-2,9
PA3934		conserved hypothetical protein	MP		-2,1		
PA3944		conserved hypothetical protein	HUU				-2,6
PA3945		hypothetical protein	HUU	2,1			-2,9
PA3956		probable short-chain dehydrogenase	HUU			-2,1	-2,2
PA3957		hypothetical protein	PE	2,1			
PA3960		hypothetical protein	HUU			-2,1	
PA3962		hypothetical protein	HUU			-2,2	
PA3979		conserved hypothetical protein	HUU		2,1		3,1
PA3981		conserved hypothetical protein	HUU			-2,1	-2,5
PA3982		conserved hypothetical protein	HUU				-2,1
PA3985		hypothetical protein	HUU	-2,0			2,5
PA3986		nicotinic acid mononucleotide	HUU			3,1	
PA4006	*nadD*	adenylyltransferase	BC				2,8
PA4007	*proA*	gamma-glutamyl phosphate reductase hypothetical protein	ABM; BC				2,1
PA4014		conserved hypothetical protein	MP				2,3
PA4015		conserved hypothetical protein	HUU			-5,4	-4,5
PA4017		probable transport protein	HUU				-2,6
PA4023		ethanolamine ammonia-lyase large subunit	TSM	3,5			-4,3
PA4024	*eutB*	prob. ethanolamine ammonia-lyase light chain	CIM	3,9			-5,1
PA4025		probable acetyltransferase	CC				-2,0
PA4026		inorganic pyrophosphatase	PE			-2,2	
PA4031	*ppa*	hypothetical protein	CIM		2,2	-2,0	
PA4041		Outer membrane protein OprG precursor	PE				-2,0
PA4067	*oprG*	hypothetical protein	MP		-2,7	6,1	3,0
PA4090		cyclic di-GMP phosphodiesterase	HUU		2,3		
PA4108		probable sensor/response regulator hybrid	CLC; MA				-2,2
PA4112		conserved hypothetical protein	TCR		-2,5		-2,2
PA4115		hypothetical protein	HUU				-2,9
PA4129		hypothetical protein	HUU			17,8	7,9
PA4129		probable sulfite or nitrite reductase	HUU				
PA4130		probable iron-sulphur protein	CIM			12,5	6,7
PA4131		conserved hypothetical protein	PE		-2,3	15,6	6,4
PA4132		cytochrome c oxidase subunit (cbb3-type)	HUU		-2,4	12,6	4,6
PA4133		hypothetical protein	EM			10,0	4,6
PA4134		probable transcriptional regulator	HUU		-3,2	15,5	5,8
PA4135		probable major facilitator superfamily tr.*	TR		2,5	-3,8	
PA4136		hypothetical protein	MP; TSM			2,4	
PA4141		ferric enterobactin transport protein FepC	HUU		-2,3	19,5	8,7
PA4158	*fepC*	hypothetical protein	TSM			2,2	
PA4205	*mexG*	efflux membrane fusion protein precursor	MP		2,7	3,0	8,8
PA4206	*mexH*	probable efflux transporter	TSM			4,4	7,8
PA4207	*mexI*	probable outer membrane protein precursor	MP; TSM			2,7	5,5
PA4208	*opmD*	prob.* phenazine-specific methyltransferase	MP; TSM				2,8
PA4209	*phzM*	probable phenazine biosynthesis protein	PE			11,0	13,4
PA4210	*phzA*	probable phenazine biosynthesis protein	SF			28,4	31,4

PA4211	*phzB*	flavin-containing monooxygenase	SF			13,8	23,3
PA4217	*phzS*	probable transporter	PE			22,7	28,0
PA4218		hypothetical protein	MP; TSM			3,8	4,2
PA4219		hypothetical protein	MP			5,8	6,0
PA4220		Fe(III)-pyochelin o.m.* receptor prc.*	HUU			7,3	6,9
PA4221	*fptA*	probable ATP-binding component of ABC tr.*	TSM			4,1	4,5
PA4222		probable ATP-binding component of ABC tr.*	TSM			6,4	5,1
PA4223		pyochelin biosynthetic protein PchG	MP; TSM			7,1	6,2
PA4224	*pchG*	pyochelin synthetase	TSM; MP		-2,0	8,5	7,5
PA4225	*pchF*	dihydroaeruginoic acid synthetase	SF ; TSM			7,5	7,6
PA4226	*pchE*	transcriptional regulator PchR	SF ; TSM			6,4	6,5
PA4227	*pchR*	pyochelin biosynthesis protein PchD	TR			2,2	2,4
PA4228	*pchD*	pyochelin biosynthetic protein PchC	SF ; TSM			4,4	4,1
PA4229	*pchC*	salicylate biosynthesis protein PchB	SF ; TSM			8,0	4,4
PA4230	*pchB*	salicylate biosynthesis isochorismate syn.*	SF ; TSM			5,6	4,3
PA4231	*pchA*	catalase	SF ; TSM			8,2	5,5
PA4236	*katA*	50S ribosomal protein L17	Ap				-2,5
PA4237	*rplQ*	DNA-directed RNA polymerase alpha chain	TPD				2,3
PA4238	*rpoA*	30S ribosomal protein S4	TRD				2,7
PA4239	*rpsD*	30S ribosomal protein S11	TPD		2,1		2,0
PA4240	*rpsK*	30S ribosomal protein S13	TPD		2,5		
PA4241	*rpsM*	50S ribosomal protein L36	TPD		2,2		2,3
PA4242	*rpmJ*	secretion protein SecY	TPD			2,1	3,4
PA4243	*secY*	50S ribosomal protein L15	MP; PSE				3,1
PA4244	*rplO*	50S ribosomal protein L30	TPD				3,3
PA4245	*rpmD*	30S ribosomal protein S5	TPD		2,1		3,0
PA4246	*rpsE*	50S ribosomal protein L18	TPD		2,1		2,6
PA4247	*rplR*	50S ribosomal protein L6	TPD		2,4		2,6
PA4248	*rplF*	30S ribosomal protein S8	TPD		2,6		2,8
PA4249	*rpsH*	30S ribosomal protein S14	TPD				2,4
PA4250	*rpsN*	50S ribosomal protein L5	TPD	-2,1			4,7
PA4251	*rplE*	50S ribosomal protein L24	TPD				3,3
PA4252	*rplX*	50S ribosomal protein L14	TPD				2,4
PA4253	*rplN*	30S ribosomal protein S17	TPD				2,7
PA4254	*rpsQ*	50S ribosomal protein L16	TPD		2,2		2,6
PA4256	*rplP*	30S ribosomal protein S3	TPD				2,4
PA4257	*rpsC*	50S ribosomal protein L22	TPD				2,3
PA4258	*rplV*	30S ribosomal protein S19	TPD				2,7
PA4259	*rpsS*	50S ribosomal protein L2	TPD				2,4
PA4260	*rplB*	50S ribosomal protein L23	TPD				2,5
PA4261	*rplW*	50S ribosomal protein L4	TPD		2,1		3,3
PA4262	*rplD*	50S ribosomal protein L3	TRD				2,6
PA4263	rplC	30S ribosomal protein S10	TPD				2,6
PA4264	rpsJ	elongation factor G	TRD	-2,1			2,9
PA4266	*fusA1*	30S ribosomal protein S7	TPD				2,6
PA4267	*rpsG*	30S ribosomal protein S12	TPD				2,4
PA4268	*rpsL*	DNA-directed RNA polymerase beta* chain	TPD				3,1
PA4269	*rpoC*	DNA-directed RNA polymerase beta chain	TRD				2,1
PA4270	*rpoB*	50S ribosomal protein L7 / L12	TRD				2,3
PA4271	*rplL*	50S ribosomal protein L10	TPD	-2,2			3,2
PA4272	*rplJ*	50S ribosomal protein L1	TPD	-2,1	2,4		2,6
PA4273	*rplA*	50S ribosomal protein L11	TPD		2,1		2,1
PA4274	*rplK*	transcription antitermination protein NusG	TPD		2,6	-2,0	2,3
PA4275	*nusG*	two-component sensor PprA	TRD				2,6
PA4293	*pprA*	hypothetical protein	TCR ;	2,9			-2,3
PA4294		two-component response regulator, PprB	HUU	4,5			-3,8
PA4296	*pprB*	TadG	TR; TCR	2,7		-2,1	-5,6
PA4297	*tadG*	hypothetical protein	MP ; MA	3,6			-4,3
PA4298		TadD	HUU	4,2			-4,6
PA4299	*tadD*	TadC	MA	4,5			-5,8
PA4300	*tadC*	TadB	MP ; MA	3,4			-3,8
PA4301	*tadB*	TadA ATPase	MP ; MA	2,8			-2,7
PA4302	*tadA*	TadZ	PSE; MA	4,5			-5,5
PA4303	*tadZ*	RcpA	MA	3,3			-4,5
PA4304	*rcpA*	RcpC	PSE; MA	3,2			-4,0
PA4305	*rcpC*	Type IVb pilin, Flp	MA	3,2			-3,5
PA4306	*flp*	chemotactic transducer PctA	MA	2,6			
PA4309	*pctA*	conserved hypothetical protein	Ap ;C	2,2			-2,2
PA4311		conserved hypothetical protein	HUU	2,3			-3,5
PA4312		formyltetrahydrofolate deformylase	HUU	2,5			-2,8
PA4314	*purU1*	hypothetical protein	NM			-2,2	
PA4324		hypothetical protein	HUU			2,3	
PA4325		hypothetical protein	HUU				2,3
PA4328		hypothetical protein	HUU		-2,2	2,6	
PA4335		hypothetical protein	HUU				-2,6
PA4337		hypothetical protein	HUU				-2,2
PA4346		conserved hypothetical protein	HUU		-2,1	2,4	
PA4348		hypothetical protein	HUU		-3,7	8,7	2,7

PA4349		conserved hypothetical protein	HUU		2,0	
PA4352		conserved hypothetical protein	HUU	-4,2	10,9	3,5
PA4357		probable ferrous iron transport protein	HUU	-3,9	6,5	
PA4358		conserved hypothetical protein	MP; TSM	-4,4	8,7	
PA4359		hypothetical protein	HUU	-3,1	5,8	2,1
PA4362		superoxide dismutase	HUU			-3,2
PA4366	*sodB*	Insulin-cleaving metalloproteinase outer	Ap	2,5	-3,6	
PA4370	*icmP*	membrane protein precursor	MP	2,0		
		ketopantoate reductase				
PA4397	*panE*	probable glutathione S-transferase	NM ; BC			-2,8
PA4401		hypothetical protein	PE	-2,2		
PA4404		CD protein FtsZ	HUU	-2,5		2,3
PA4407	*ftsZ*	conserved hypothetical protein	CD			-3,7
PA4421		30S ribosomal protein S9	HUU	-2,2		
PA4432	*rpsI*	50S ribosomal protein L13	TPD			2,6
PA4433	*rplM*	ATP sulphurylase GTP-binding subunit	TPD	2,3		2,4
PA4442	*cysN*	ATP sulphurylase small subunit	CIM			3,1
PA4443	*cysD*	conserved hypothetical protein	CIM ABM			3,5
PA4465		hypothetical protein	HUU			-2,0
PA4467		superoxide dismutase	MP	-3,5	14,2	3,7
PA4468	*sodM*	hypothetical protein	Ap	-2,5	6,3	2,5
PA4469		fumarate hydratase FumC1	HUU	-3,1	11,1	3,4
PA4470	*fumC*	hypothetical protein	EM	-2,8	13,4	4,6
PA4471		rod shape-determining protein MreB	HUU		5,3	3,4
PA4481	*mreB*	Glu-tRNA(Gln) amidotransferase subunit C	CLC; CD	2,2	-2,1	
PA4482	*gatC*	probable two-component sensor	TPD	2,4		
PA4494		hypothetical protein	TCR	-2,3		
PA4495		probable binding protein com.*t of ABC tr.*	HUU			-2,4
PA4496		probable metallopeptidase	TSM	2,7	-2,2	-5,0
PA4498		probable transcriptional regulator	TPD			2,6
PA4499		probable binding protein com.*of ABC tr.*	TR		-2,2	
PA4500		Glycine-glutamate dipeptide porin OpdP	TSM	2,2		
PA4501	*opdD*	probable binding protein com.* of ABC tr.*	TSM; MP	3,6		
PA4502		probable permease of ABC transporter	TSM	3,6		
PA4503		probable permease of ABC transporter	MP; TSM	2,2		
PA4504		prob.*ATP-binding com.* of ABC dipeptide tr.*	MP; TSM	2,3		
PA4506		hypothetical protein	TSM	3,2		
PA4507		probable transcriptional regulator	MP	2,2		
PA4508		probable Ctransducer	TR		-2,2	-2,4
PA4520		hypothetical protein	Ap ;C		-2,1	
PA4531		hypothetical protein	HUU	-3,7	4,1	
PA4535		hypothetical protein	HUU			-2,5
PA4536		hypothetical protein	HUU			-2,7
PA4537		competence protein ComL	HUU		-2,4	-2,0
PA4545	*comL*	30S ribosomal protein S20	CLC	2,0	-2,0	
PA4563	*rpsT*	50S ribosomal protein L27	CIM;TPD	4,5	-2,7	3,2
PA4567	*rpmA*	50S ribosomal protein L21	TPD	-2,3		4,0
PA4568	*rplU*	hypothetical protein	TPD	2,0		2,2
PA4570		probable cytochrome c	HUU		11,9	5,4
PA4571		hypothetical protein	EM	-2,1	2,7	
PA4573		hypothetical protein	HUU	-2,0	2,8	
PA4577		hypothetical protein	HUU	-4,4	7,6	
PA4578		cytochrome c551 peroxidase precursor	HUU		-4,3	-2,6
PA4587	*ccpR*	probable outer membrane protein precursor	EM	-2,2	2,4	
PA4589		protein activator	MP; TSM		-2,8	-3,6
PA4590	*pra*	probable outer membrane protein precursor	TSM; CC	3,3	-26,0	-14,7
PA4592		serine hydroxymethyltransferase	HUU		-2,1	
PA4602	*glyA3*	conserved hypothetical protein	ABM	-12,8	-2,4	-2,6	2,1
PA4605		conserved hypothetical protein	HUU			-2,8
PA4606		hypothetical protein	Ap	-2,2		-2,4
PA4607		hypothetical protein	HUU	-2,1		-2,5
PA4608		hypothetical protein	HUU			-2,5
PA4610		hypothetical protein	HUU	-2,8	3,8	
PA4611		hypothetical protein	HUU	-3,0	4,5	
PA4625		hypothetical protein	HUU			2,3
PA4637		still frameshift hypothetical protein	HUU			2,9
PA4641		hypothetical protein	HUU	2,6		-5,2
PA4648		hypothetical protein	HUU	6,9		-11,7
PA4649		hypothetical protein	HUU	4,6		-7,4
PA4650		probable pili assembly chaperone	HUU	3,4		-5,1
PA4651		hypothetical protein	MA; CHP	4,9		-8,7
PA4652		hypothetical protein	HUU			-2,0
PA4653		probable major facilitator superfamily tr.*	HUU	2,3		-3,0
PA4654		hypothetical protein	MP; TSM		-4,8	-3,1
PA4657		hypothetical protein	HUU			-2,2
PA4658		probable transcriptional regulator	HUU			-2,6
PA4659		ribose-phosphate pyrophosphokinase	TR			-2,1
PA4670	*prs*	probable ribosomal protein L25	CC ; NM	3,1	-2,3	2,5

PA4671		peptidyl-tRNA hydrolase	Ap ; TPD	2,6		3,3
PA4672		hypothetical protein	TPD			2,1
PA4680		hypothetical protein	HUU		-5,6	-8,6
PA4681		hypothetical protein	HUU		-4,4	-7,8
PA4682		hypothetical protein	HUU	2,1	-3,2	-7,2
PA4683		hypothetical protein	HUU			-2,1
PA4689		ketol-acid reductoisomerase	HUU			-2,2
PA4694	*ilvC*	acetolactate synthase isozyme III small sub.*	BC; ABM		-5,1	-3,8
PA4695	*ilvH*	acetolactate synthase large subunit	BC; ABM		-4,5	-3,0
PA4696	*ilvI*	hypothetical protein	BC; ABM		-4,8	-2,9
PA4702		hypothetical protein	HUU	2,2		-3,1
PA4703		Heme-transport protein, PhuT	HUU			-3,4
PA4708	*phuT*	probable hemin degrading factor	TSM		3,0	2,9
PA4709		hypothetical protein	PE		3,1	3,6
PA4713		conserved hypothetical protein	HUU	-3,0	4,2	
PA4717		acetyl-coenzyme A synthetase	HUU			-2,1
PA4733	*acsB*	hypothetical protein	CC ; CIM		-2,0	-2,6
PA4735		hypothetical protein	HUU			-3,2
PA4737		30S ribosomal protein S15	HUU	-2,5	2,2	
PA4741	*rpsO*	tRNA pseudouridine 55 synthase	TPD	-2,5		2,8
PA4742	*truB*	ribosome-binding factor A	TPD			2,1
PA4743	*rbfA*	translation initiation factor IF-2	TPD ; Ap			2,1
PA4744	*infB*	triosephosphate isomerase	TPD			2,3
PA4748	*tpiA*	hypothetical protein	CIM ; EM	-2,0		2,3
PA4754		SmpB protein	MP			2,1
PA4768	*smpB*	cyclic di-GMP phosphodiesterase	TPD	-2,1		2,2
PA4781		hypothetical protein	TR	2,4		-4,7
PA4782		probable transcriptional regulator	HUU		-2,1	
PA4784		probable transcriptional regulator	TR			-2,5
PA4787		hypothetical protein	TR		-2,6	
PA4788		conserved hypothetical protein	HUU			-2,0
PA4792		hypothetical protein	HUU		2,2	
PA4801		FdhE protein	HUU	-2,3		2,4
PA4809	*fdhE*	nitrate-inducible formate dehyd.*,gamma sub.	EM			-2,1
PA4810	*fdnI*	nitrate-inducible formate dehyd.*, beta sub.*	EM			-2,9
PA4811	*fdnH*	formate dehydrogenase-O, major subunit	EM	2,3		-3,7
PA4812	*fdnG*	lipase LipC	EM	2,4		-4,0
PA4813	*lipC*	3-dehydroquinate dehydratase	FPM	2,2	-3,3	-7,0
PA4846	*aroQ1*	biotin carboxyl carrier protein (BCCP)	ABM	2,4		
PA4847	*accB*	biotin carboxylase	FPM	2,1		
PA4848	*accC*	ribosomal protein L11 methyltransferase	FPM	2,3		
PA4850	*prmA*	conserved hypothetical protein	TPD		-2,1	
PA4852		DNA-binding protein Fis	HUU	-2,2		3,7
PA4853	*fis*	hypothetical protein	TR; DRR			2,1
PA4872		conserved hypothetical protein	HUU		-2,4	
PA4874		probable bacterioferritin	HUU			-3,9
PA4880		probable transmembrane sensor	CIM			-2,0
PA4895		probable sigma-70 factor, ECF subfamily	MP ; TR		2,7	
PA4896		probable short-chain dehydrogenase	TR		2,2	
PA4907		hypothetical protein	PE		-3,4	-2,7
PA4908		branched chain amino acid ABC tr.*ATP b.p.*	HUU	2,2		
PA4910		prob. permease of ABC b.-chain* a.a.* tr.*	TSM	2,9	2,3	-2,6	-3,3
PA4911		b.-chain* amino acid ABC tr.* m.p.*	MP; TSM	2,6		-3,1
PA4912		prob.* binding protein component of ABC tr.*	MP; TSM	3,2	2,6	-3,9	-4,9
PA4913		probable transcriptional regulator	TSM	3,2	2,5	-5,0	-6,2
PA4914		probable Ctransducer	TR			-2,4
PA4915		hypothetical protein	Ap ;C		-2,2		-5,4
PA4916		hypothetical protein	HUU			2,0
PA4917		NH3-dependent NAD synthetase	HUU		2,5	2,5
PA4920	*nadE*	hypothetical protein	ABM; BC		-2,7	-2,1
PA4921		azurin precursor	HUU			-2,9
PA4922	*azu*	conserved hypothetical protein	EM		2,0	
PA4925		50S ribosomal protein L9	HUU	2,1		-3,4
PA4932	*rplI*	hypothetical protein	TPD	-2,2	2,5	3,0
PA4933		30S ribosomal protein S18	MP	-2,2		3,0
PA4934	*rpsR*	30S ribosomal protein S6	TPD		2,7	3,0
PA4935	*rpsF*	30S ribosomal protein S6	TPD	-2,0	2,1	
PA4935	*rpsF*	oligoribonuclease	TPD			2,9
PA4951	*orn*	hypothetical protein	TRD			-2,4
PA4958		hypothetical protein	HUU			-2,4
PA4965		conserved hypothetical protein	HUU	-2,1		
PA4969		hypothetical protein	HUU		-2,2	-2,1
PA4972		probable outer membrane protein precursor	HUU			-2,5
PA4974		hypothetical protein	PSE		-2,4	
PA4985		conserved hypothetical protein	HUU			-2,8
PA4998		branched-chain amino acid transferase	HUU		-2,1	
PA5013	*ilvE*	pyruvate dehydrogenase	ABM		-2,4	
PA5015	*aceE*	peptide methionine sulfoxide reductase	ABM; EM		-2,1	-2,6

PA5018	*msrA*	hypothetical protein	TPD			-2,0	
PA5027		conserved hypothetical protein	HUU		-2,3	3,4	
PA5028		Type 4 fimbrial biogenesis o.m. p.* PilQ prc.*	HUU			-2,2	
PA5040	*pilQ*	type 4 fimbrial biogenesis protein PilP	MA			-2,3	-2,2
PA5041	*pilP*	50S ribosomal protein L31	MA			-2,0	
PA5049	*rpmE*	heat shock protein HslU	TPD	-2,4			4,3
PA5054	*hslU*	polyhydroxyalkanoate synthesis protein PhaF	CHP			-2,0	
PA5060	*phaF*	probable permease of ABC transporter	CIM				-2,5
PA5075		prob.* binding protein component of ABC tr.*	MP; TSM			-2,9	
PA5076		N-formylglutamate amidohydrolase	TSM		2,4	-3,1	
PA5091	*hutG*	Prob.* histidine/phenylalanine ammonia-lyase	ABM		2,3		
PA5093		probable amino acid permease	PE			2,0	
PA5097		histidine ammonia-lyase	MP; TSM			2,2	3,5
PA5098	*hutH*	probable transporter	ABM				2,8
PA5099		urocanase	MP; TSM		2,2		2,5
PA5100	*hutU*	hypothetical protein	ABM		2,5		4,4
PA5101		hypothetical protein	HUU				-2,1
PA5103		conserved hypothetical protein	HUU				-2,7
PA5104		histidine utilization repressor HutC	HUU			2,8	
PA5105	*hutC*	conserved hypothetical protein	TR			2,8	
PA5106		outer membrane lipoprotein Blc	HUU			8,4	6,0
PA5107	*blc*	hypothetical protein	MP				-2,3
PA5108		esterase EstA	HUU				-2,2
PA5112	*estA*	regulatory protein TypA	SF ; FPM			-2,8	-3,7
PA5117	*typA*	thiazole biosynthesis protein ThiI	Ap				2,1
PA5118	*thiI*	hypothetical protein	BC				2,1
PA5122		hypothetical protein	HUU			-2,9	
PA5137		hypothetical protein	HUU			-2,5	
PA5139		conserved hypothetical protein	HUU			-2,4	
PA5148		probable short-chain dehydrogenase	HUU		2,1	-2,5	
PA5150		probable ATP-binding component of ABC tr.*	PE			4,1	
PA5152		a.a* ABC tr.*periplasmic binding protein	TSM			-2,8	-3,6
PA5153		probable permease of ABC transporter	TSM	2,0		-6,7	-7,2
PA5154		a.a* ABC transporter membrane protein	MP; TSM			-4,5	-4,1
PA5155		probable c4-dicarboxylate-binding protein	MP ;TSM			-3,6	-3,5
PA5167		probable dicarboxylate transporter	MP; TSM	2,6		-3,1	-5,5
PA5168		probable C4-dicarboxylate transporter	MP; TSM	2,4			-3,9
PA5169		arginine/ornithine antiporter	MP; TSM	2,5		-2,8	-5,3
PA5170	*arcD*	arginine deiminase	MP; ABM	2,7	-2,0	2,6	-2,1
PA5171	*arcA*	ornithine carbamoyltransferase, catabolic	ABM	3,3	-2,6	2,8	-3,1
PA5172	*arcB*	carbamate kinase	ABM	3,4	-2,6	2,7	-3,1
PA5173	*arcC*	probable beta-ketoacyl synthase	ABM	3,2	-2,5	3,8	-2,1
PA5174		conserved hypothetical protein	FPM			2,1	3,4
PA5178		hypothetical protein	HUU				-2,4
PA5182		hypothetical protein	MP			2,2	
PA5191		phosphoenolpyruvate carboxykinase	HUU	2,2			-3,4
PA5192	*pckA*	hypothetical protein	CC ; EM				2,3
PA5196		ribosomal protein S6 modification protein	HUU				-2,3
PA5197	*rimK*	N-acetylglutamate synthase	TRD				-3,1
PA5204	*argA*	probable phosphate transporter	ABM			2,0	2,0
PA5207		conserved hypothetical protein	MP; TSM		-2,1	2,0	
PA5208		probable secretion pathway ATPase	HUU		-3,1	2,9	
PA5210		hypothetical protein	PSE				-2,0
PA5212		glycine cleavage system protein P1	HUU	5,2			-4,6
PA5213	*gcvP1*	hypothetical protein	CIM;ABM	2,4			-3,5
PA5219		hypothetical protein	MP				2,4
PA5220		probable permease of ABC transporter	HUU				2,6
PA5230		prob.*ATP-binding/permease fusion ABC tr.*	MP; TSM		-2,3	2,4	
PA5231		conserved hypothetical protein	MP; TSM		-2,8	2,6	
PA5232		hypothetical protein	HUU		-2,4	2,8	
PA5233		probable oxidoreductase	HUU			-2,1	
PA5234		conserved hypothetical protein	PE			-2,4	
PA5245		alginate regulatory protein AlgP	HUU			-2,0	-2,0
PA5253	*algP*	alginate biosynthesis regulatory protein AlgR	TR			-2,2	-2,7
PA5261	*algR*	hypothetical protein	SF ; TR				-3,4
PA5270		hypothetical protein	HUU			-2,0	
PA5271		nucleoside diphosphate kinase regulator	HUU	2,0		-2,1	-4,2
PA5274	*rnk*	conserved hypothetical protein	TR	-2,2		-3,0	
PA5279		hypothetical protein	HUU			-2,2	
PA5289		xanthine phosphoribosyltransferase	HUU			-2,2	
PA5298		probable transcriptional regulator	NM		2,2	-2,5	
PA5301		catabolic alanine racemase	TR			-2,0	
PA5302	*dadX*	conserved hypothetical protein	ABM	2,1		6,4	
PA5303		D-amino acid dehydrogenase, small subunit	HUU	4,1	-2,5	20,9	2,0
PA5304	*dadA*	conserved hypothetical protein	EM; ABM	3,4		6,5	
PA5305		conserved hypothetical protein	HUU				-2,1
PA5306		probable aldehyde dehydrogenase	HUU				-2,4
PA5312		prob.* pyridoxal-dependent aminotransferase	PE				-2,2

PA5313		hypothetical protein	PE	2,1			-3,3
PA5314		50S ribosomal protein L33	HUU	2,1			-2,5
PA5315	*rpmG*	50S ribosomal protein L28	TPD	-2,1			4,0
PA5316	*rpmB*	RNA polymerase omega subunit	TPD	-2,0	2,1		3,1
PA5337	*rpoZ*	conserved hypothetical protein	TRD				2,0
PA5339		OxyR	HUU			-2,1	
PA5344	*oxyR*	hypothetical protein	TR ; MA			-2,3	
PA5347		probable DNA-binding protein	HUU			-2,6	
PA5348		Rubredoxin 1	DRR			-3,4	-2,1
PA5351	*rubA1*	conserved hypothetical protein	CC				2,6
PA5352		glycolate oxidase subunit GlcF	HUU	5,8		-3,8	-24,1
PA5353	*glcF*	glycolate oxidase subunit GlcE	CIM ; CC	5,2		-3,3	-18,4
PA5354	*glcE*	glycolate oxidase subunit GlcD	CIM ; CC	3,3		-4,5	-16,8
PA5355	*glcD*	transcriptional regulator GlcC	CIM ; CC	3,9		-3,2	-13,8
PA5356	*glcC*	hypothetical protein	TR	2,1			-4,5
PA5359		probable ATP-binding component of ABC tr.*	HUU				-3,1
PA5376		probable permease of ABC transporter	TSM	2,1			-3,5
PA5377		hypothetical protein	MP; TSM	2,8			-4,3
PA5378		L-serine dehydratase	HUU	2,9		-2,8	-6,0
PA5379	*sdaB*	GbdR	ABM				-2,2
PA5380	*gbdR*	conserved hypothetical protein	TR	2,2	2,7	-2,7	-2,2
PA5395		hypothetical protein	HUU				-2,7
PA5396		hypothetical protein	HUU	3,0			-5,1
PA5397		DgcA, Dimethylglycine catabolism	HUU	2,2			-3,0
PA5398	*dgcA*	DgcB, Dimethylglycine catabolism	ABM				-2,7
PA5399	*dgcB*	hypothetical protein	ABM				-2,1
PA5401		hypothetical protein	HUU				-2,1
PA5408		hypothetical protein	HUU				-2,2
PA5409		GbcA	MP				-2,2
PA5410	*gbcA*	GbcB	ABM	4,0		-2,5	-8,0
PA5411	*gbcB*	hypothetical protein	ABM	2,5		-2,3	-4,3
PA5414		serine hydroxymethyltransferase	HUU			-2,0	
PA5415	*glyA1*	sarcosine oxidase beta subunit	ABM	2,5			-2,5
PA5416	*soxB*	sarcosine oxidase delta subunit	ABM	3,1			-5,2
PA5417	*soxD*	sarcosine oxidase gamma subunit	ABM	3,4			-4,5
PA5419	*soxG*	formyltetrahydrofolate deformylase	ABM	2,7			-3,7
PA5420	*purU2*	glutathione-independent formaldehyde dehyd.	NM				-2,6
PA5421	*fdhA*	hypothetical protein	CIM	3,0			-4,9
PA5422		hypothetical protein	HUU				-2,0
PA5423		conserved hypothetical protein	HUU				-2,3
PA5424		alcohol dehydrogenase	MP		-2,4		-2,5
PA5427	*adhA*	conserved hypothetical protein	EM ; CC		-3,0	4,9	
PA5442		hypothetical protein	MP			-2,3	
PA5446		glycosyltransferase WbpZ	HUU		-2,1	14,4	9,4
PA5447	*wbpZ*	glycosyltransferase WbpY	CLC				-2,1
PA5448	*wbpY*	glycosyltransferase WbpX	CLC			-4,6	-2,7
PA5449	*wbpX*	ABC subunit of A-band LPS efflux transporter	CLC			-3,4	-3,2
PA5450	*wzt*	membrane subunit of A-band LPS efflux tr.*	CLC			-4,6	-3,0
PA5451	*wzm*	phosphomannose isomerase/GDP-mannose	CLC; MP			-3,2	-2,7
PA5452	*wbpW*	WbpW	CLC			-3,0	-2,6
PA5453	*gmd*	GDP-mannose 4,6-dehydratase oxidoreductase Rmd	CLC			-3,3	-3,3
PA5454	*rmd*	hypothetical protein	CLC			-3,6	-2,9
PA5455		hypothetical protein	HUU			-2,9	-2,2
PA5456		hypothetical protein	HUU			-3,9	-2,3
PA5457		hypothetical protein	HUU			-3,7	-2,1
PA5458		hypothetical protein	MP			-2,6	
PA5459		hypothetical protein	HUU			-4,0	-2,9
PA5461		hypothetical protein	HUU			-2,0	
PA5462		hypothetical protein	HUU			-2,3	
PA5475		hypothetical protein	HUU		-5,1	16,9	
PA5475		proton-glutamate symporter	HUU				3,2
PA5479	*gltP*	hypothetical protein	MP; TSM				3,3
PA5482		class II (cobalamin-dependent) ribonucleotide-	MP	2,0			
PA5496	*nrdJb*	diphosphate reductase sub.*, NrdJb hypothetical protein	NM		-2,1	2,4	
PA5502		D-methionine ABC tr.* membrane protein	HUU				2,0
PA5504		probable short-chain dehydrogenase	MP; TSM				2,1
PA5521		probable glutamine synthetase	PE				-2,8
PA5522		probable aminotransferase	PE				-2,1
PA5523		probable transcriptional regulator	PE				-2,6
PA5525		hypothetical protein	TR				-2,7
PA5526		hypothetical protein	HUU				-2,1
PA5527		hypothetical protein	HUU				-2,3
PA5542		conserved hypothetical protein	PE			-2,2	-2,6
PA5544		conserved hypothetical protein	MP				-2,4
PA5545		conserved hypothetical protein	HUU			-3,0	-4,3
PA5546		GlmR transcriptional regulator	PE	2,2			-3,4

PA5550	*glmR*	ATP synthase epsilon chain	TR			2,1
PA5553	*atpC*	ATP synthase beta chain	EM	-2,4		3,6
PA5554	*atpD*	ATP synthase gamma chain	EM			2,2
PA5555	*atpG*	ATP synthase alpha chain	EM			2,7
PA5556	*atpA*	ATP synthase delta chain	EM			2,3
PA5557	*atpH*	ATP synthase B chain	EM			2,7
PA5558	*atpF*	atp synthase C chain	EM	-2,1		2,9
PA5559	*atpE*	ATP synthase A chain	EM			2,1
PA5560	*atpB*	ATP synthase protein I	EM			2,1
PA5561	*atpI*	ribonuclease P protein component	MP ; EM	-2,2		3,1
PA5569	*rnpA*	ribonuclease P protein component	TPD		2,3	
PA5569	*rnpA*	50S ribosomal protein L34	TPD	-2,1		2,7
PA5570	*rpmH*		CIM			2,4

Continued

Gene ID[a]	Gene name[a]	PCFC[a,b]	FC			
			W4/ M4	W12/ M12	M4/ M12	W4/ W14
tRNA Ala		NR	-2,2			2,1
tRNA Asn	PA3139.1/PA4541.3	NR	-2,5			
tRNA Cys	PA2581.1	NR	-2,2			
tRNA Gly		NR	-2,2			2,2
tRNA His		NR	-2,5			2,4
tRNA Ile		NR	-3,3			3,1
tRNA Lys		NR	-2,9			2,4
tRNA Phe	PA5149.1	NR	-2,1			
tRNA Pro	PA2736.1/PA3031.1/ PA4541.2/	NR	-2,2			
tRNA Ser	PA2852.1/PA0905.1/ PA1013.1/PA2603.1/	NR	-2,6	-2,5		
tRNA Trp		NR				2,3
tRNA Val	PA2775.1/PA3094.3/PA3262.2	NR	-2,5			

[a] Gene ID, gene name, function and PCFC are according to the "Pseudomonas Genome Database" (Winsor *et al.*, 2011).

[b] Abbreviations used for PseudoCAP Function Class (PCFC) are as following Adaptation, Protection (AP); Amino acid biosynthesis and metabolism (ABM); Antibiotic resistance and susceptibility (ARS); Biosynthesis of cofactors, prosthetic groups and carriers (BC);Carbon compound catabolism (CC); Cell wall, LPS, capsule (CLC); Central intermediary metabolism (CIM); Chaperones & heat shock proteins (CHP); Chemotaxis (C); Cell division (CD); DNA replication, recombination, modification and repair (DRR); Energy metabolism (EM); Fatty acid and phospholipid metabolism (FPM); Hypothetical, unclassified, unknown (HUU); Membrane proteins (MP); Motility & Attachment (MA); Non-coding RNA gene (NR); Nucleotide biosynthesis and metabolism (NM); Protein secretion/export apparatus (PSE); Putative enzymes (PE); Related to phage, transposon, or plasmid (RPT); Secreted Factors (toxins, enzymes, alginate) (SF); Transcriptional regulators (TR); Transcription, RNA processing and degradation (TRD); Translation, post-translational modification, degradation (TPD); Transport of small molecules (TSM); Two-component regulatory systems (TCR) and Unclassified genome space (UGS).

* Other abbreviations used are amino acid (a.a.), basal-body (b-b.), branched-chain (b.-chain), binding protein (b.p.), biosynthesis protein (bio. p.), component (com.), dehydrogenase (dehyd.), dependent (dep.), electron (e.), factor (fc.), flavoprotein (fp.), membrane-bound (m.-b.), membrane protein (m.p.), outer membrane (o.m.), oxidase (ox.), protein (p.), protein synthase (p.s.), precursor (prc.), probable (prob.), subunit (sub.), synthase (syn.), transporter (tr.) and transferase (transf.)

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